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Figure 1

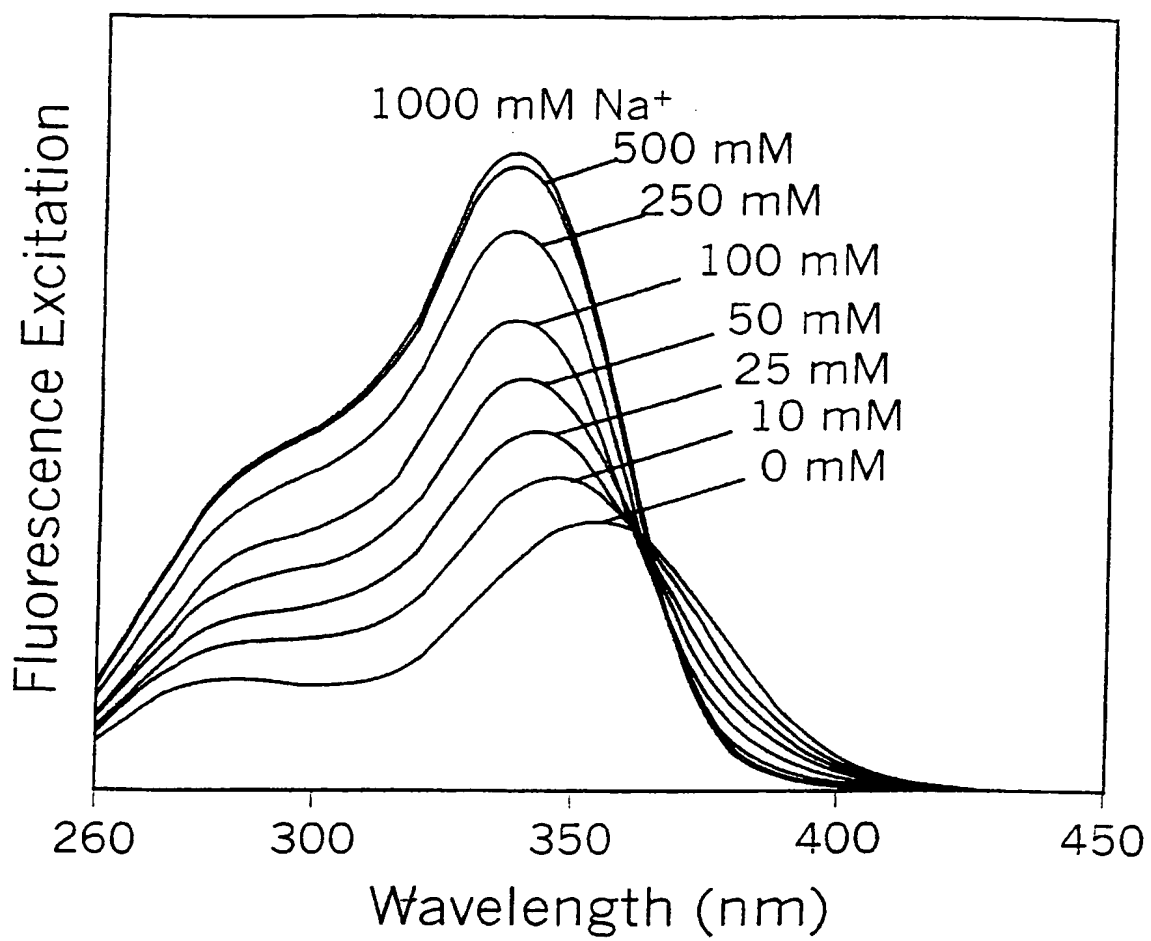


Figure 2

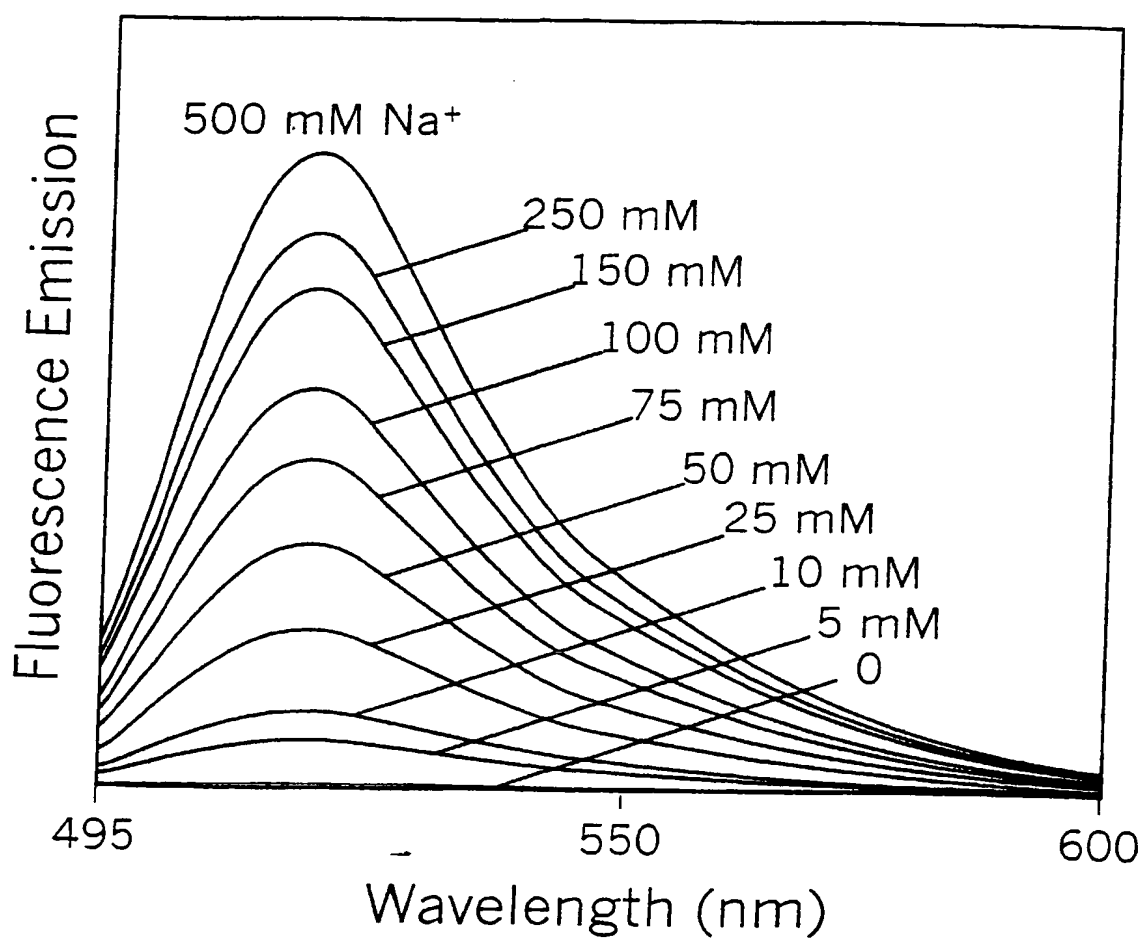
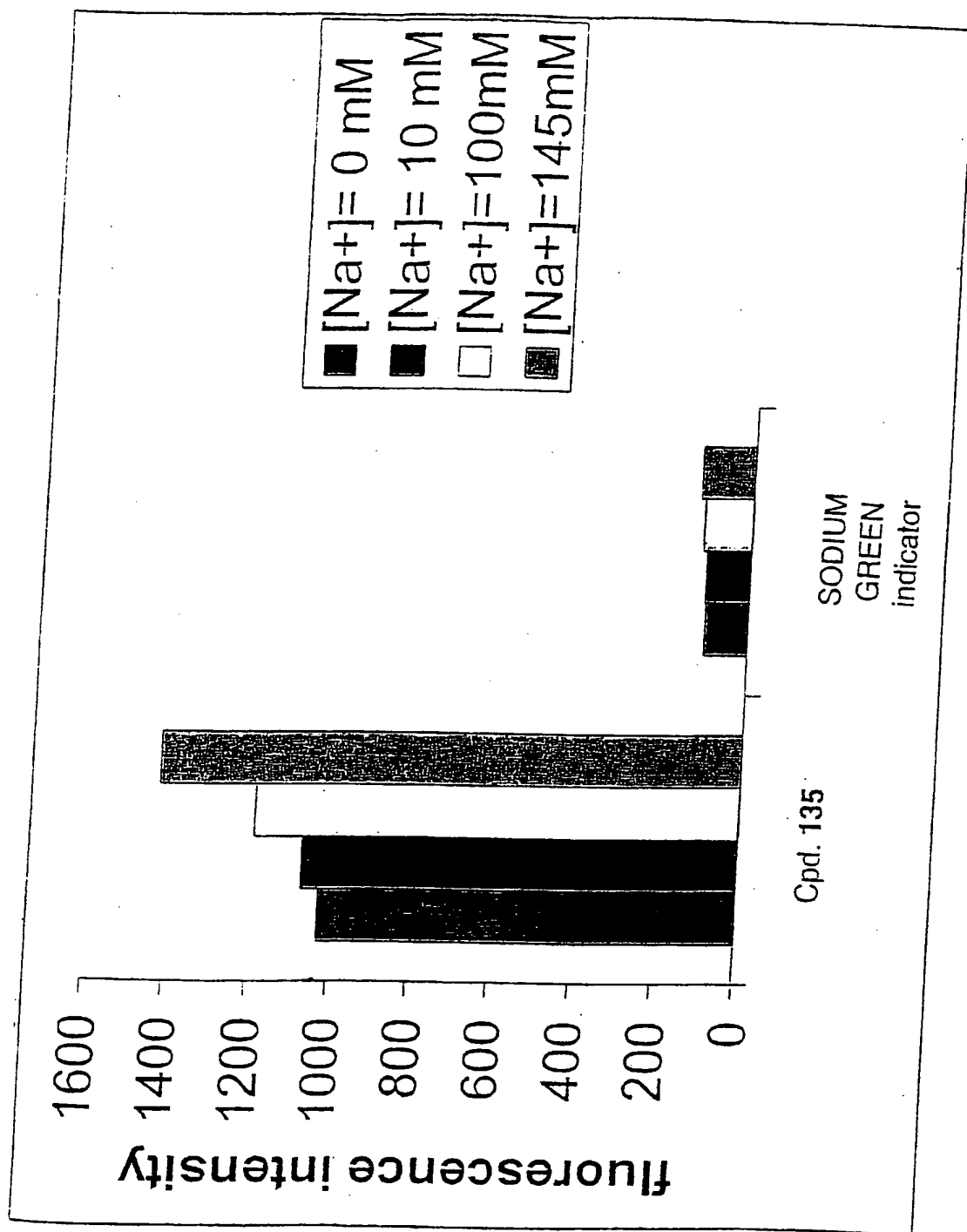


Figure 3



CROWN ETHER DERIVATIVES

FIELD OF THE INVENTION

5 The invention relates to derivatives of crown ether chelators, including chromophoric and fluorescent derivatives, that are useful for chelating metal cations. Where the chelator is labeled with a fluorophore, it is an indicator useful for the detection, discrimination and quantification of metal cations. The chelators are optionally substituted one or more times with a chemically reactive group or a conjugated substance, such as a biological or nonbiological
10 polymer, or a lipid.

BACKGROUND

15 Metal ions play an important role in biological systems. Cells utilize metal ions for a wide variety of functions, such as regulating enzyme activity, protein structure, cellular signaling, as catalysts, as templates for polymer formation and as regulatory elements for gene transcription. Metal ions can also have a deleterious effect when present in excess of bodily requirements or capacity to excrete. A large number of natural and synthetic materials are known to selectively or non-selectively bind to or chelate metal ions. Ion chelators are
20 commonly used in solution for in vivo control of ionic concentrations and detoxification of excess metals, and as in vitro buffers. When bound to a fluorophore, ion chelators are typically used as optical indicators of ions and are useful in the analysis of cellular microenvironments or dynamic properties of proteins, membranes and nucleic acids.

25 Such indicators are also useful for measuring ions in extracellular spaces; in vesicles; in vascular tissue of plants and animals; biological fluids such as blood and urine; in fermentation media; in environmental samples such as water, soil, waste water and seawater; and in chemical reactors. Optical indicators for ions are important for qualitative and quantitative determination of ions, particularly in living cells. Fluorescent indicators for metal cations also
30 permit the continuous or intermittent optical determination of these ions in living cells, and in solutions containing the ions.

 A variety of fluorescent indicators that are useful for the detection of biologically relevant soluble free metal ions (such as Ca^{2+} , Mg^{2+} and Zn^{2+}) have been described that utilize
35 oxygen-containing anionic or polyanionic chelators to bind to metal ions. In particular, fluorescent indicators utilizing a polycarboxylate BAPTA chelator have been previously described (U.S. Patent No.: 4,603,209 to Tsien et al. (1986); U.S. Patent No. 5,049,673 to Tsien et al. (1991); U.S. Patent No. 4,849,362 to DeMarinis et al. (1989); U.S. Patent No. 5,453,517 to Kuhn et al. (1995); U.S. Patent No. 5,501,980 to Malekzadeh et al. (1996); U.S. Patent No.
40 5,459,276 to Kuhn et al. (1995); U.S. Patent No. 5,501,980 to Katerinopoulos et al. (1996); U.S.

Patent No. 5,459,276 to Kuhn et al. (1995) (all incorporated by reference). Some fluorescent indicators selective for Li^+ , Na^+ and K^+ in aqueous or organic solution have also been described, based on the chemical modification of crown ethers (U.S. Patent No. 5,134,232 to Tsien et al. (1992); U.S. Patent No. 5,405,975 to Kuhn et al. (1995); both incorporated by reference).

In general, a useful property for metal ion indicators is the ability to detect and/or quantify a selected metal ion in the presence of other metal ions. Discrimination of Ca^{2+} , Na^+ and K^+ ions in the presence of other metal ions is particularly useful for certain biological or environmental samples. For most biological applications, it is essential that the indicators be effective in aqueous solutions. It is also useful that indicators for biological applications be relatively insensitive to pH changes over the physiological range (pH 6-8) and sensitive to ion concentrations in the physiological range (for sodium, a K_a of about 5 mM to about 20 mM). It is also beneficial if the indicator absorbs and emits light in the visible spectrum where biological materials have low intrinsic absorbance or fluorescence.

Also useful are chelators that possess a chemically reactive functional group, so that the chelating group can be attached to polymers for use in remote sensing of ions or enhancing the solubility or localization of the optical sensor. Many chelators bind to intracellular proteins, altering the chelator's metal binding properties. In addition, due to their relatively small size, they are readily sequestered non-selectively in intracellular vesicles, further limiting their effectiveness. One means of circumventing these problems is to attach the desired crown ether to a large, water-soluble polysaccharide, such as dextran or FICOL, by means of modification of the polysaccharide to allow covalent attachment of the indicator. Dextrans and FICOLs are especially suitable for this application, as they are low cost, optically transparent above about 250 nm and available in multiple ranges of molecular weights. Furthermore, polysaccharides and their conjugates are reasonably compatible with most biological materials and do not interact significantly with intracellular components. Although fluorescent polysaccharides have been previously described, as have indicator conjugates of dextrans, none possess the advantageous properties of the indicator conjugates of the current invention.

The crown ether chelators of the invention show significant ability to discriminate between metal ions under physiological conditions, particularly Ca^{2+} , Na^+ and K^+ ions. This selectivity can be tailored by careful selection of crown ether substituents. The compounds of the invention are typically soluble in aqueous solutions.

The compounds of the invention that act as indicators for target ions absorb and emit light in the visible spectrum and possess significant utility as a means of detecting and quantifying certain metal ion levels in living cells, biological fluids or aqueous solutions. Upon binding the target ion in the chelating moiety of the indicator, the optical properties of the

attached fluorophore are generally affected in a detectable way, and this change is correlated with the presence of the ion according to a defined standard. Compounds having relatively long wavelength excitation and emission bands can be used with a variety of optical devices and require no specialized (quartz) optics, such as are required by indicators that are excited or that emit at shorter wavelengths. These indicators are suitable for use in fluorescence microscopy, flow cytometry, fluoroscopy, or any other application that currently utilize fluorescent metal ion indicators.

BRIEF DESCRIPTION OF THE DRAWINGS

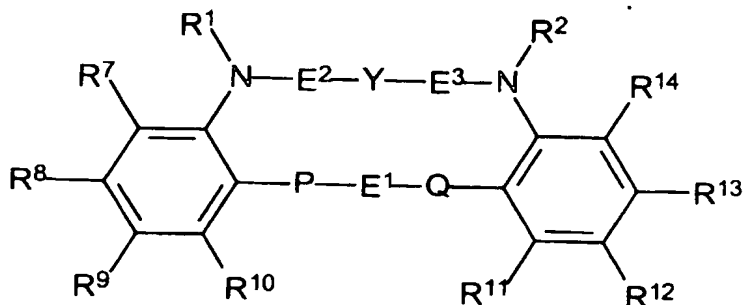
Fig 1. The Na⁺-dependent fluorescence excitation spectra of Compound 167 in a series of solutions containing 0 to 1000 mM free Na⁺, with fluorescence emission monitored at 510 nm (as described in Example 71).

Fig 2. The Na⁺-dependent fluorescence emission spectra of Compound 132 in a series of solutions containing 0 to 500 mM free Na⁺, with excitation at 488 nm (as described in Example 71).

Figure 3: A comparison of the intracellular sodium response of SODIUM GREEN tetraacetate indicator (Molecular Probes, Inc.) or Compound 134. At every intracellular Na⁺ concentration, Compound 134 demonstrates a stronger fluorescence intensity than SODIUM GREEN sodium indicator at the same concentration (as described in Example 72).

SUMMARY OF THE INVENTION AND DESCRIPTION OF THE PREFERRED EMBODIMENTS

The compounds of the invention have the formula



The heteroatoms P and Q are independently selected from O, S, or NR³, where each R³ is independently H or an alkyl having 1-6 carbons. In one aspect of the invention P and Q are both O.

The heteroatom Y is O, S, or NR^4 , where R^4 is H, a $\text{C}_1\text{-C}_{18}$ alkyl, or an aryl or heteroaryl ring system. The R^4 alkyl or ring system substituent is optionally substituted one or more times by halogen, azido, nitro, nitroso, amino, alkylamino having 1-6 carbons, dialkylamino having 2-12 carbons, cyano, or R^4 is substituted one or more times by a $\text{C}_1\text{-C}_6$ alkyl or $\text{C}_1\text{-C}_6$ alkoxy that is itself optionally substituted one or more times by halogen, amino, hydroxy, $-(\text{SO}_2)\text{-R}^{15}$, $-(\text{SO}_2)\text{-O-R}^{15}$, $-(\text{C=O})\text{-R}^{15}$, $-(\text{C=O})\text{-O-R}^{15}$, or $-(\text{C=O})\text{-NR}^{17}\text{R}^{18}$. Alternatively, R^4 is, or is substituted by $-\text{L-R}_x$, $-\text{L-Sc}$, or $-\text{L-DYE}$.

Each R^{16} is independently H or $\text{C}_1\text{-C}_6$ alkyl. Each R^{16} is H, a $\text{C}_1\text{-C}_6$ alkyl, a benzyl, or forms an ester (e.g., R^{16} is an alpha-acyloxyalkyl, a t-butyldimethylsilyl, or any other biologically compatible esterifying group). Additionally, any R^{16} is a biologically compatible salt. R^{17} and R^{18} are independently H or $\text{C}_1\text{-C}_6$ alkyl or $\text{C}_1\text{-C}_6$ carboxyalkyl, or an alpha-acyloxyalkyl, a t-butyldimethylsilyl, or any other biologically compatible esterifying group, or a biologically compatible salt; or R^{17} and R^{18} when taken in combination form a 5- or 6-membered aliphatic ring that optionally incorporates an oxygen atom. In addition, one or more of a R^{15} , R^{16} , R^{17} , or R^{18} is permitted to be $-\text{L-R}_x$, $-\text{L-Sc}$, or $-\text{L-DYE}$.

Each L is independently a covalent linkage. Each R_x is independently a reactive functional group. Each Sc is independently a conjugated substance. Each DYE is independently a moiety that maximally absorbs light at a wavelength greater than 320 nm.

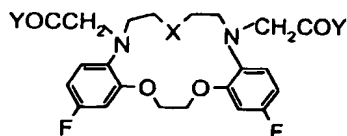
In one aspect of the invention, P and Q are O, and Y is NR^4 . In another aspect of the invention, P, Q, and Y are each O. Careful selection of the nature of the P, Q, and Y heteroatoms permits the moderation of the selectivity and binding affinity of the resulting crown ether.

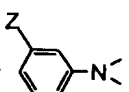
E^1 , E^2 , and E^3 each independently have the formula $-(\text{CR}^5)_n-$, or $-(\text{C}(\text{O})\text{CH}_2)_n-$, where $n = 2\text{-}4$. Each R^5 is independently H or methyl, or the R^5 moieties on adjacent carbon atoms of each chain, when taken in combination, form a 5- or 6-membered aliphatic ring. For a given E moiety, each R^5 is typically H and n is 2. Where n is 2 for each E moiety, the resulting compound is known as a 15-crown-5 crown ether, having 15 atoms in the chelating ring itself, of which 5 are heteroatoms.

The amine substituents R^1 and R^2 are independently $\text{C}_1\text{-C}_{18}$ alkyl, or $\text{C}_7\text{-C}_{18}$ arylalkyl. Where R^1 or R^2 is alkyl or arylalkyl, it is optionally substituted one or more times by halogen, azido, nitro, nitroso, amino, hydroxy, $\text{C}_1\text{-C}_6$ alkylamino, $\text{C}_2\text{-C}_{12}$ dialkylamino, cyano, or by an aryl or heteroaryl ring system, or by $-(\text{SO}_2)\text{-R}^{15}$, $-(\text{SO}_2)\text{-O-R}^{15}$, $-(\text{C=O})\text{-R}^{15}$, $-(\text{C=O})\text{-O-R}^{15}$, or $-(\text{C=O})\text{-NR}^{17}\text{R}^{18}$, where R^{15} , R^{16} , R^{17} , and R^{18} are as defined above; or by a $\text{C}_1\text{-C}_6$ alkyl or $\text{C}_1\text{-C}_6$ alkoxy that is itself optionally substituted one or more times by halogen, amino, hydroxy,

(
 $-(\text{SO}_2)-\text{R}^{16}$, $-(\text{SO}_2)-\text{O}-\text{R}^{16}$, $-(\text{C}=\text{O})-\text{R}^{16}$, $-(\text{C}=\text{O})-\text{O}-\text{R}^{16}$, or $-(\text{C}=\text{O})-\text{NR}^{17}\text{R}^{18}$. Alternatively, R^1 and R^2 are optionally $-\text{L}-\text{R}_x$, $-\text{L}-\text{Sc}$, or $-\text{L}-\text{DYE}$.

Compounds of the following formula are not claimed per se:



where Y is OH or OEt and X is $-\text{O}-$, or  where Z is $-\text{NO}_2$, NH_2 or $\text{N}(\text{CH}_2\text{COY})_2$.

5

For purposes of this invention, an aryl or aromatic ring system means a substituent containing 1 to 4 unsaturated rings (each ring containing 6 conjugated carbon atoms and no heteroatoms) that are optionally fused to each other or bonded to each other by carbon-carbon single bonds, and is optionally substituted as described below. Specific examples of an aryl ring system include, but are not limited to, substituted or unsubstituted derivatives of phenyl, biphenyl, *o*-, *m*-, or *p*-terphenyl, 1-naphthyl, 2-naphthyl, 1-, 2-, or 9-anthryl, 1-, 2-, 3-, 4-, or 9-phenanthrenyl and 1-, 2- or 4-pyrenyl. Preferably an aryl substituent is phenyl, substituted phenyl, naphthyl or substituted naphthyl.

15 For purposes of this invention, a heteroaryl or heteroaromatic ring system means a 5- or 6-membered unsaturated ring that is optionally fused to an additional six-membered aromatic ring(s), or is fused to one 5- or 6-membered unsaturated ring containing one or more heteroatoms, and is optionally substituted as defined below. Each heteroaromatic ring contains at least 1 and as many as 3 heteroatoms that are selected from the group consisting of O, N or S in any combination. Specific examples of a heteroaryl ring system include, but are not limited to, substituted or unsubstituted derivatives of 2- or 3-furanyl; 2- or 3-thienyl; N-, 2- or 3-pyrrolyl; 2- or 3-benzofuranyl; 2- or 3-benzothieryl; N-, 2- or 3-indolyl; 2-, 3- or 4-pyridyl; 2-, 3- or 4-quinolyl; 1-, 3-, or 4-isoquinolyl; 2-, 4-, or 5-(1,3-oxazolyl); 2-benzoxazolyl; 2-, 4-, or 5-(1,3-thiazolyl); 2-benzothiazolyl; 3-, 4-, or 5-isoxazolyl; N-, 2-, or 4-imidazolyl; N-, or 2-benzimidazolyl; 1- or 2-naphthofuranyl; 1- or 2-naphthothieryl; N-, 2- or 3-benzindolyl; 2-, 3-, or 4-benzoquinolyl; 1-, 2-, 3-, or 4-acridinyl. Preferably the heteroaryl substituent is substituted or unsubstituted 4-pyridyl, 2-thienyl, 2-pyrrolyl, 2-indolyl, 2-oxazolyl, 2-benzothiazolyl or 2-benzoxazolyl. More preferably, the heteroaryl substituent is 2-thienyl or 2-pyrrolyl.

30

Any aryl or heteroaryl ring system is unsubstituted or optionally and independently substituted by any synthetically accessible and chemically stable combination of substituents

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that are independ ntly H, halogen, cyano, sulfo, alkali or ammonium salt of sulfo, nitro, carboxy, alkyl, perfluoroalkyl, alkoxy, alkylthio, amino, monoalkylamino, dialkylamino or alkylamido, typically the alkyl portions of which contain 18 or fewer carbons.

- 5 Where the R¹ and R² substituents are not -L-R_x, -L-Sc, or -L-DYE, they are typically both alkyl that is substituted one or more times by carboxylic acids, by carboxylic acid esters, by carboxylic acid amides, or by cyano. Where R¹ and R² incorporate carboxylic acid esters, they are optionally selected to be esters that are readily cleaved by intracellular esterase enzymes, such as alpha-acyloxyalkyl esters (typically acetoxymethyl esters). Alternatively, R¹
10 and R² incorporate carboxylic acid esters that are typically not cleaved, such as esters of lower

alcohols (for example methyl or ethyl esters). In one embodiment, at least one of R¹, R², and R⁷-R¹⁴ incorporates multiple carboxylic acids or carboxylic acid esters. Selection of the precise nature of R¹ and R² can greatly affect the selectivity and binding selectivity and affinity of the resulting compound (see Table 1).

5

The benzo substituents R⁷-R¹⁴ are independently H, halogen, azido, nitro, nitroso, amino, cyano; or -L-R_x, -L-Sc, or -L-DYE; or C₁-C₆ alkyl or C₁-C₆ alkoxy that is itself optionally substituted one or more times by halogen, amino, hydroxy, -(SO₂)-R¹⁵, -(SO₂)-O-R¹⁵, -(C=O)-R¹⁵, -(C=O)-O-R¹⁶, or -(C=O)-NR¹⁷R¹⁸.

10

Alternatively, two adjacent substituents of R⁷-R¹⁴, when taken in combination, form a fused six-membered benzo moiety, which is optionally substituted one or more times by a halogen, azido, nitro, nitroso, amino, cyano, -L-R_x, -L-Sc, or -L-DYE; or by a C₁-C₆ alkyl or C₁-C₆ alkoxy, which is itself optionally substituted one or more times by halogen, amino, hydroxy, -(SO₂)-R¹⁵, -(SO₂)-O-R¹⁵, -(C=O)-R¹⁵, -(C=O)-O-R¹⁶, or -(C=O)-NR¹⁷R¹⁸.

15

In one aspect of the invention, two adjacent substituents of R⁷-R¹⁴, when taken in combination with each other, and with the aromatic ring they are bound to, form a fused DYE.

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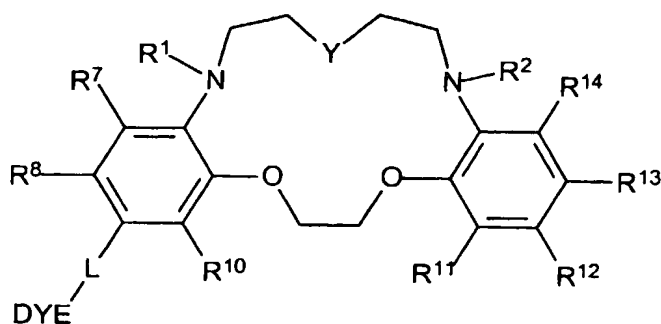
The compounds of the invention are substituted by at least one -L-DYE, -L-R_x or -L-Sc at one or more of R¹, R², R⁴, and R⁷-R¹⁴; or two of R⁷-R¹⁴, taken in combination, form a fused DYE. In one embodiment, the compound of the invention is substituted by exactly one -L-DYE moiety, which is bound at R¹, R², an R⁴, or one of R⁷-R¹⁴, or is a fused DYE moiety at two adjacent substituents of R⁷-R¹⁴. The DYE moiety is typically bound at one of R⁷-R¹¹, preferably at R⁹, or is bound at R⁴ where Y is NR⁴. In one embodiment, compounds that are substituted by exactly one -L-DYE moiety are optionally further substituted by -L-R_x or -L-Sc, typically at R¹, R², R⁴, or one of R⁷-R¹⁴.

25

In another embodiment, the compound of the invention is substituted by exactly two DYE moieties, which may be the same or different, and may be bound by a covalent linkage L or fused to the crown ether chelate. In one embodiment of the invention, a first -L-DYE moiety is bound at one of R⁷-R¹⁰, while the second -L-DYE moiety is bound at one of R¹¹-R¹⁴. Typically, the first -L-DYE moiety is bound at R⁹, while the second -L-DYE moiety is bound at R¹², or a DYE moiety is fused at R⁸ and R⁹ and additionally at R¹² and R¹³.

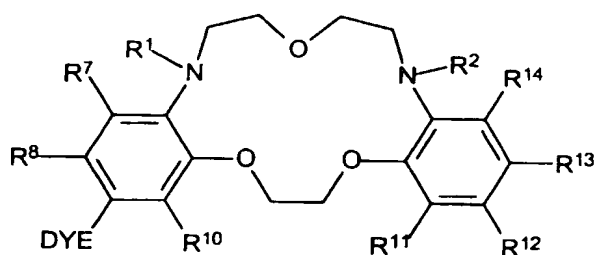
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In one aspect of the invention, the compound of the invention has the formula



where Y is either O or NR⁴; and the DYE moiety is an indole, a coumarin, a stilbene, a xanthene, or a polyazaindacene. In this embodiment, preferably the DYE moiety is a xanthene, a polyazaindacene, or an oxazine.

In one aspect of the invention, the compounds of the invention are fluorescent indicators having the following structure:

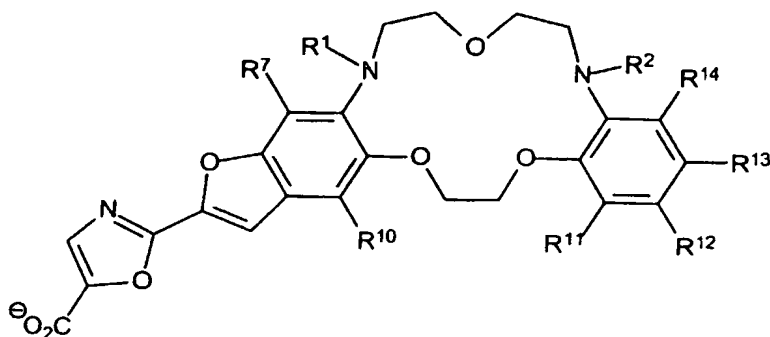


These indicators typically exhibit a low fluorescence quantum efficiency in the absence of metal ions. However, in the presence of increasing metal ion concentration the fluorescence quantum efficiency rises dramatically. For example, selected indicators of this family exhibit a fluorescence signal increase of over 100-times between zero and a saturating sodium concentration. Other selected indicators of the invention exhibit a shift of the wavelength of the absorption (excitation) maximum, emission maximum, or both, upon binding the target ion.

In one embodiment of this aspect, R¹ and R² are C₁-C₆ alkyl that are substituted one or more times by cyano, an aryl or heteroaryl ring system, or by -(C=O)-O-R¹⁶ or -(C=O)-NR¹⁷R¹⁸, where R¹⁶ is H, a C₁-C₆ alkyl, a benzyl, a biologically compatible esterifying group, or a biologically compatible salt; and R¹⁷ and R¹⁸ are independently H, C₁-C₆ alkyl, C₁-C₆ carboxyalkyl, an alpha-acyloxymethyl, or a biologically compatible salt. The substituents R⁷, R⁸, R¹⁰, and R¹¹-R¹⁴, are independently H, chloro, bromo, fluoro, nitro, amino, or cyano; or C₁-C₆ alkyl or C₁-C₆ alkoxy that is itself optionally substituted by halogen, amino, hydroxy, -(SO₂)-R¹⁵, -(SO₂)-O-R¹⁵, -(C=O)-R¹⁵, -(C=O)-O-R¹⁶, or -(C=O)-NR¹⁷R¹⁸. The DYE moiety is a polyazaindacene, an oxazine, or a xanthene, which is optionally substituted one or more times

by halogen, nitro, sulfo, cyano, an aryl or heteroaryl ring system, or benzo, or alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, acyl, or carboxylic acids or carboxylic acid esters, the alkyl portions of which contain fewer than 20 carbons.

- 5 In another aspect of the invention, the compounds of the invention are fluorescent indicators having the following structure:

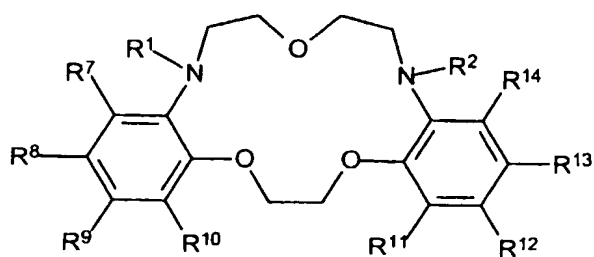


- 10 This class of indicators, in which at least one of the aromatic rings of the crown ether portion of the indicator is also incorporated in the DYE moiety, typically exhibit ratiometric fluorescence excitation changes in response to changing metal ion concentration. That is, there is a shift in the excitation maximum wavelength in the presence of increasing metal ion concentrations.

15

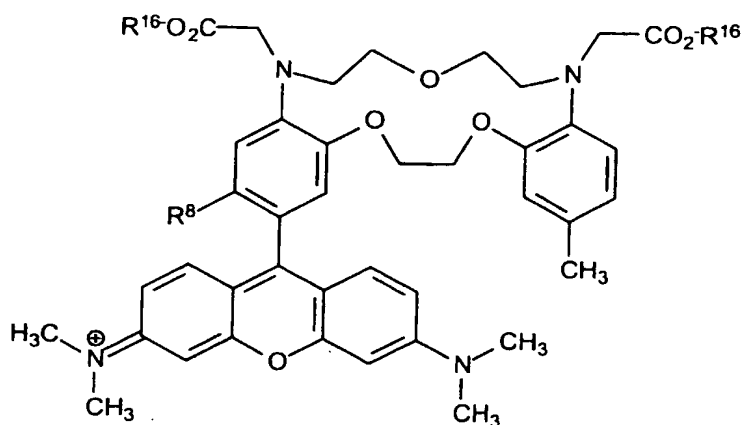
- In one embodiment of this aspect, R¹ and R² are C₁-C₆ alkyl that are substituted one or more times by cyano, an aryl or heteroaryl ring system, or by -(C=O)-O-R¹⁶ or -(C=O)-NR¹⁷R¹⁸, where R¹⁶ is H, a C₁-C₆ alkyl, a benzyl, a biologically compatible esterifying group, or a biologically compatible salt; and R¹⁷ and R¹⁸ are independently H, C₁-C₆ alkyl, C₁-C₆ carboxyalkyl, an alpha-acyloxymethyl, or a biologically compatible salt. The substituents R⁷, R¹⁰, and R¹¹-R¹⁴, are independently H, chloro, bromo, fluoro, nitro, amino, or cyano; or C₁-C₆ alkyl or C₁-C₆ alkoxy that is itself optionally substituted by halogen, amino, hydroxy, -(SO₂)-R¹⁵, -(SO₂)-O-R¹⁵, -(C=O)-R¹⁵, -(C=O)-O-R¹⁶, or -(C=O)-NR¹⁷R¹⁸.

- 25 The nature of the R¹ and R² substituents in large part determines the response of the indicators to particular target ions. For example, where the crown ether derivatives have the formula



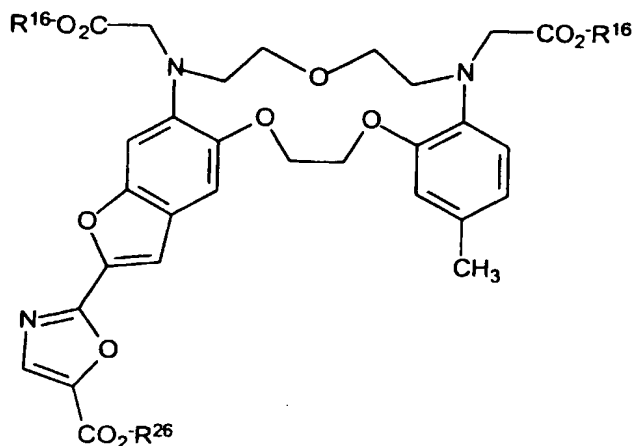
where R^1 and R^2 are each methoxycarbonylmethyl ^{they} selectively typically bind sodium ions with a dissociation constant (K_d) of approximately 20-100 mM, and are relatively insensitive to the presence of potassium ions. In particular, the sodium ion K_d values typically rise less than about 10% when measured in the presence of 100 mM potassium ion.

An additional selected embodiment of the invention has the formula

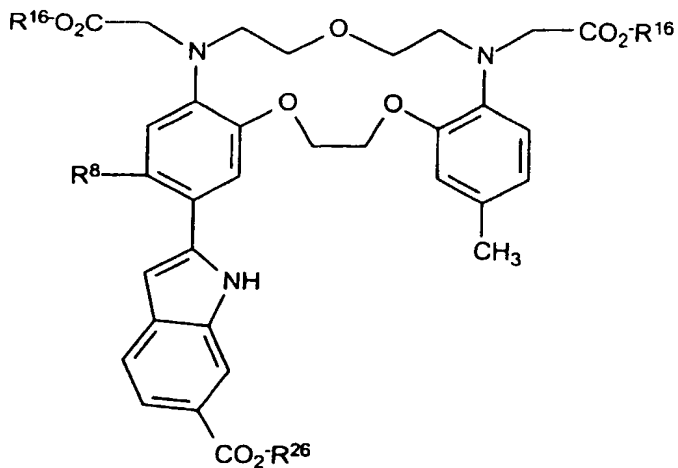


where R^8 and R^{16} are defined as above.

An additional selected embodiment of the invention has the formula

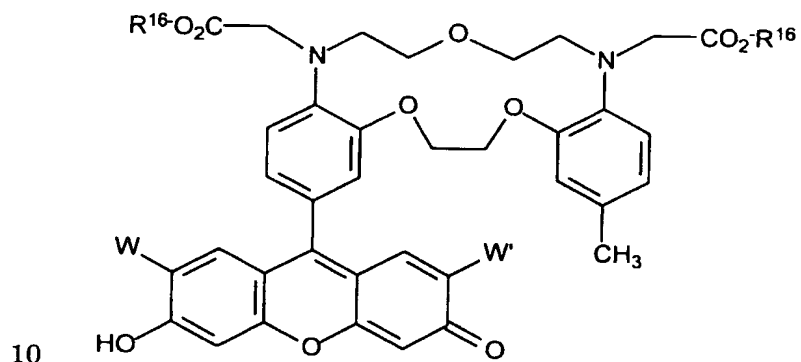


or the formula



- 5 where R^8 and R^{16} are defined as above, and R^{26} is H, a C_1 - C_6 alkyl, a benzyl, or is an alpha-acyloxyalkyl or a t-butyldimethylsilyl or other biologically compatible esterifying group, or is a biologically compatible salt.

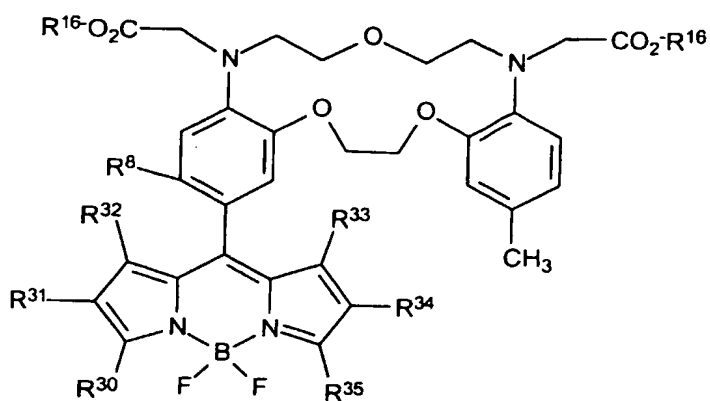
An additional selected embodiment of the invention has the formula



10 where R^{16} is defined as above, and W and W' are independently F or Cl.

An additional selected embodiment of the invention has the formula

15



where R^8 and R^{16} are defined as above, and R^{30} - R^{35} are independently hydrogen, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, or acyl, wherein the alkyl portions of each contain fewer than 20 carbons; or aryl or heteroaryl ring system; or adjacent substituents R^{31} and R^{32} , and adjacent substituents R^{33} and R^{34} , when taken in combination form a fused benzo ring that is optionally substituted one or more times by hydrogen, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, alkylthio, alkylamido, amino, monoalkylamino or dialkylamino wherein the alkyl portions of each contain fewer than 20 carbons.

In an additional aspect of the invention, the compound of the invention further comprises a metal ion that is associated and/or complexes within the crown ether chelate portion of the compound. The metal ion is optionally a monocation (such as Li^+ , Na^+ , K^+ , Rb^+ , or Cs^+), a dication (such as Ca^{2+} , Zn^{2+} , or Mg^{2+}), or a polycation (such as Tb^{3+} or Eu^{3+}).

The DYE Moiety

The DYE moiety is any chemical moiety that exhibits an absorption maximum beyond 320 nm, that is bound to the crown ether chelate by a covalent linkage L, or that is fused to the crown ether chelate. The covalent linkage L is either a single covalent bond, or a combination of stable chemical bonds, as described in greater detail below. The covalent linkage binding the DYE moiety to the crown ether chelator is typically a single bond, but optionally incorporates 1-20 nonhydrogen atoms selected from the group consisting of C, N, O, P, and S. In one embodiment, L is or incorporates a phenylene or a 2-carboxy-substituted phenylene.

Typically the DYE moiety contains one or more aromatic or heteroaromatic rings, that are optionally substituted one or more times by a variety of substituents, including without limitation, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, acyl, aryl or heteroaryl ring system, benzo, or other substituents typically present on chromophores or fluorophores known in the art.

In one aspect of the invention, the DYE moiety has an absorption maximum beyond 480 nm. In a particularly useful embodiment, the DYE moiety absorbs at or near 488 nm to 514 nm (particularly suitable for excitation by the output of the argon-ion laser excitation source) or near 546 nm (particularly suitable for excitation by a mercury arc lamp). The DYE moiety may be a chromophore, resulting in a compound that acts as a chromogenic indicator, or more preferably, DYE is additionally a fluorophore, resulting in a compound that is a fluorescent indicator. Preferably, binding a target ion within the crown ether chelate results in a detectable optical response. As used herein, a detectable optical response means a change in, or occurrence of, an optical signal that is detectable either by observation or instrumentally, such a change in absorption (excitation) wavelength, fluorescence emission wavelength, or fluorescence emission intensity.

Where the detectable response is a fluorescence response, it is typically a change in fluorescence, such as a change in the intensity, excitation or emission wavelength distribution of fluorescence, fluorescence lifetime, fluorescence polarization, or a combination thereof. Preferably, the detectable optical response upon binding the target metal ion is a change in fluorescence intensity that is greater than approximately 10-fold, more preferably greater than 50-fold. In another aspect, the detectable optical response upon binding the target metal ion is a shift in maximal excitation or emission wavelength that is greater than about 20 nm, more preferably greater than about 30 nm. Sodium and potassium indicators of the type that exhibit significant emission shifts have not been previously described.

A wide variety of chemically reactive fluorescent dyes that may be suitable for incorporation into the compounds of the invention are already known in the art (see for example MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS, Sixth Ed., Richard P. Haugland, ed. (1996), and Seventh Ed., Richard P. Haugland, ed. (1999) on CD-ROM; BIOPROBES 32 (December 1999); BIOPROBES 33 (February 2000); BIOPROBES 34 (May 2000); and BIOPROBES 35 (November 2000)). The spectral properties of candidate dyes in solution or when conjugated to proteins such as IgG are known or are readily measured using an absorption spectrometer or a spectrofluorometer.

In one aspect of the invention, the DYE moiety is a pyrene, an anthracene, a naphthalene, an acridine, a stilbene, an indole or benzindole, an oxazole or benzoxazole, a thiazole or benzothiazole, a 4-amino-7-nitrobenz-2-oxa-1,3-diazole (NBD), a cyanine, a carbocyanine, a carbostyryl, a porphyrin, a salicylate, an anthranilate, an azulene, a perylene, a pyridine, a quinoline, a coumarin (including hydroxycoumarins and aminocoumarins and fluorinated derivatives thereof (as described in U.S. Patent No. 5,830,912 (1998), incorporated by reference), a polyazaindacene (e.g., US Patents 4,774,339 to Haugland, et al. (1988); 5,187,288 to Kang, et al. (1993); 5,248,782 to Haugland, et al. (1993); 5,274,113 to Kang, et al. (1993); and 5,433,896 to Kang, et al. (1995), all incorporated by reference), a xanthene, an

oxazine or a benzoxazine, a carbazine (US Patent 4,810,636 to Corey (1989), incorporated by reference), or a phenalenone or benzphenalenone (US Patent 4,812,409 Babb et al. (1989), incorporated by reference). In another aspect of the invention, the DYE moiety is a carbazine, an oxazine, a coumarin, a pyrene, a xanthene, a naphthalene, a phenalenone, or a polyazaindacene. As used herein, oxazines include resorufins, aminooxazinones, diaminoxazines, and their benzo-substituted analogs.

Where the DYE moiety is a xanthene, the synthetic dye is optionally a fluorescein, a rhodol (US Patent 5,227,487 to Haugland, et al. (1993), incorporated by reference), or a rhodamine. As used herein, fluorescein includes benzo- or dibenzofluoresceins, seminaphthofluoresceins, or naphthofluoresceins. Similarly, as used herein rhodol includes seminaphthorhodafluors (U.S. Patent No. 4,945,171 to Haugland, et al. (1990), incorporated by reference). Fluorinated xanthene dyes have been described previously as possessing particularly useful fluorescence properties (Int. Publ. No. WO 97/39064, Molecular Probes, Inc. (1997), U.S. Patent No. 6,162,931 to Gee et al. (2000), incorporated by reference).

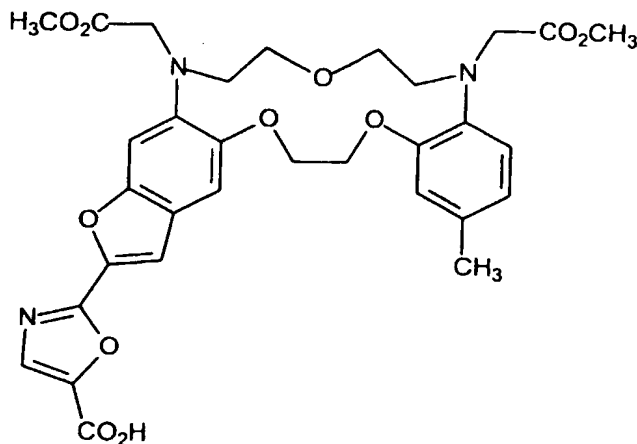
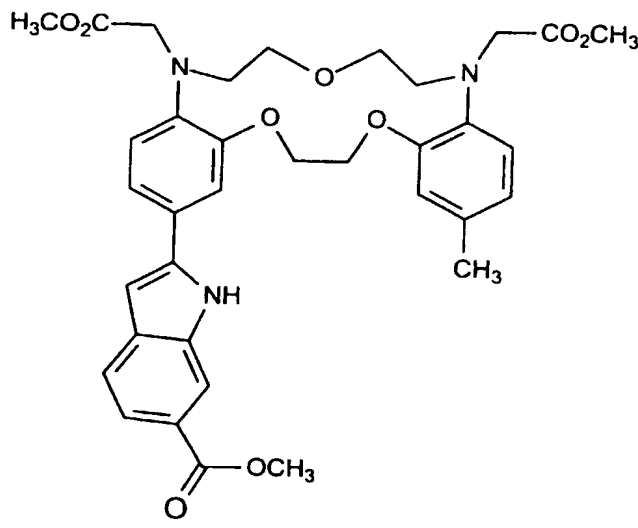
Alternatively, the DYE moiety is a xanthene that is bound via an L that is a single covalent bond at the 9-position of the xanthene. Preferred xanthenes include derivatives of 3*H*-xanthen-6-ol-3-one bound at the 9-position, derivatives of 6-amino-3*H*-xanthen-3-one bound at the 9-position, or derivatives of 6-amino-3*H*-xanthen-3-imine bound at the 9-position.

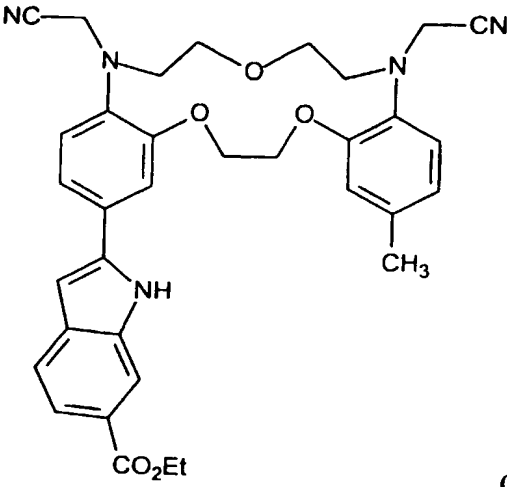
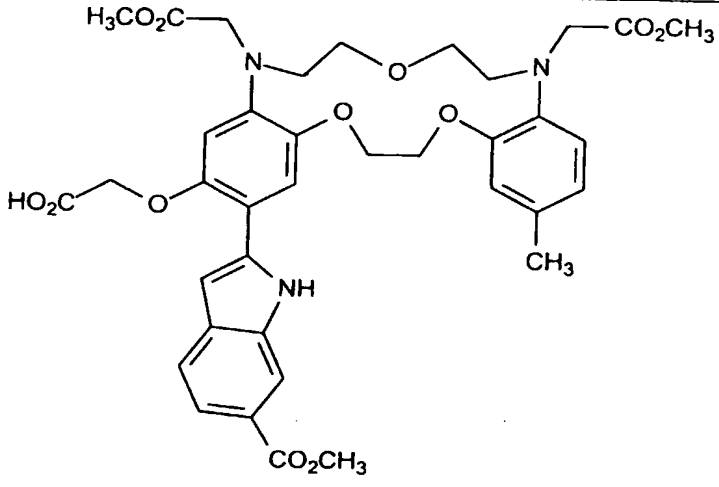
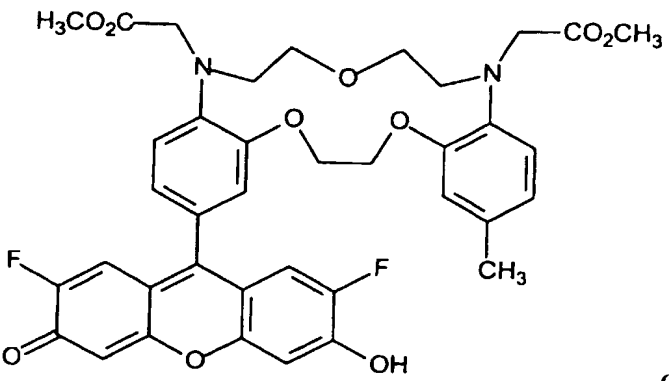
Selected sulfonated DYE moieties also exhibit advantageous properties, and include sulfonated pyrenes, coumarins, carbocyanines, and xanthenes (as described in U.S. Patent No. 5,132,432 to Haugland et al., (1992); U.S. Patent No. 5,696,157 to Wang et al. (1997); U.S. Patent No. 5,268,486 patent to Waggoner et al. (1993); U.S. Patent No. 6,130,101 to Mao et al. (2000); all incorporated by reference). Sulfonated pyrenes and coumarins are typically excited at wavelengths below about 450 nm.

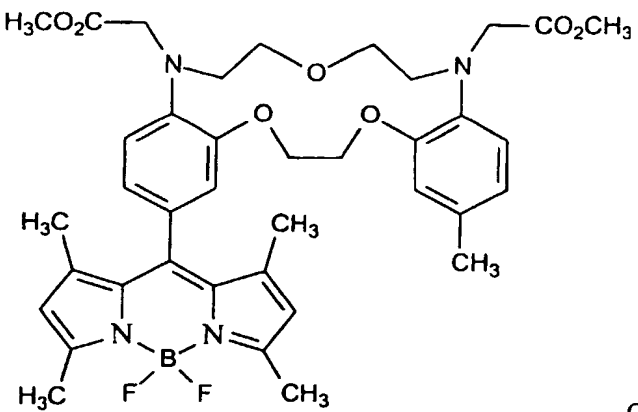
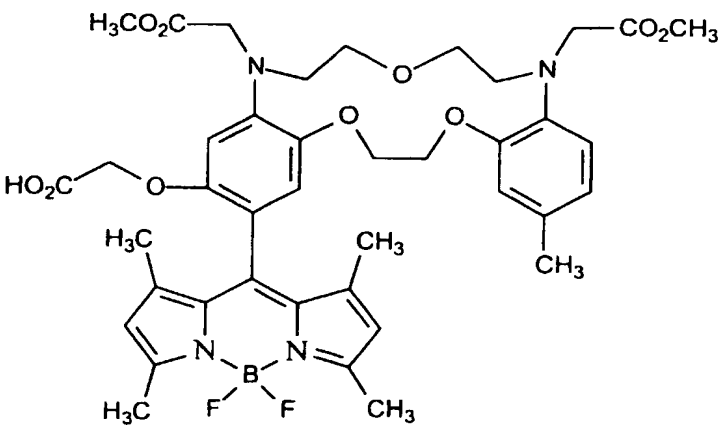
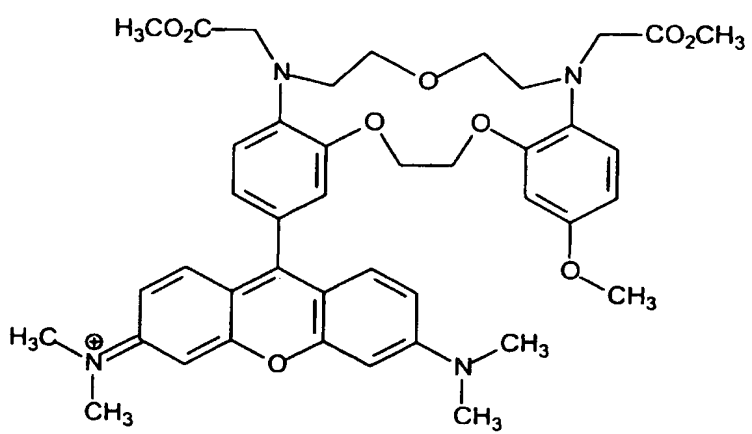
As described above, in one aspect of the invention, two adjacent substituents of R⁷-R¹⁴, when taken in combination with each other, and with the aromatic ring they are bound to, form a fused DYE. In one aspect of the invention, the fused DYE is a carbocyanine dye (as in Wang et al., J. WUHAN UNIV 2, 73 (1991)). In another embodiment, two adjacent ring substituents taken in combination form DYE moiety that is a fused benzofuran or heteroaryl- or carboxyheteroaryl-substituted benzofuran fluorophore, or a fused 6-membered unsaturated lactone, or benzazole-substituted lactone. Where the DYE moiety is fused to the compound of the invention, it is preferably fused R⁸ and R⁹, or at R¹² and R¹³.

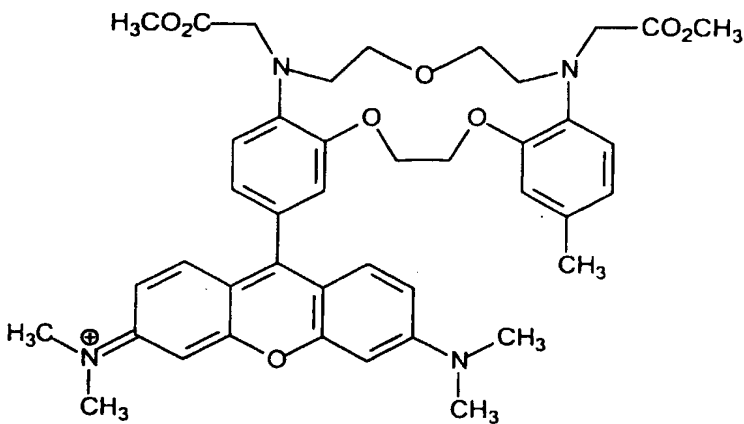
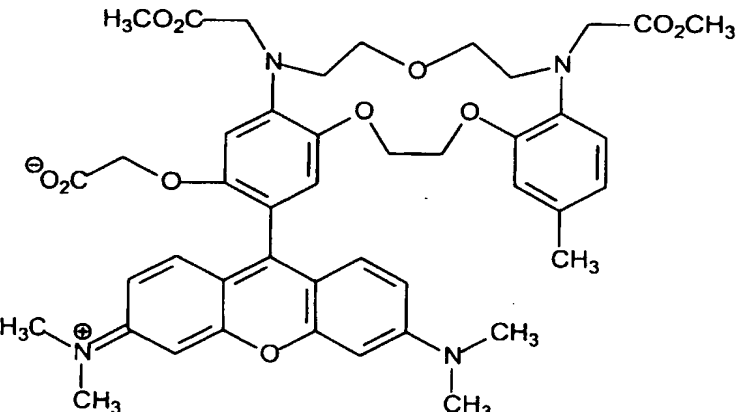
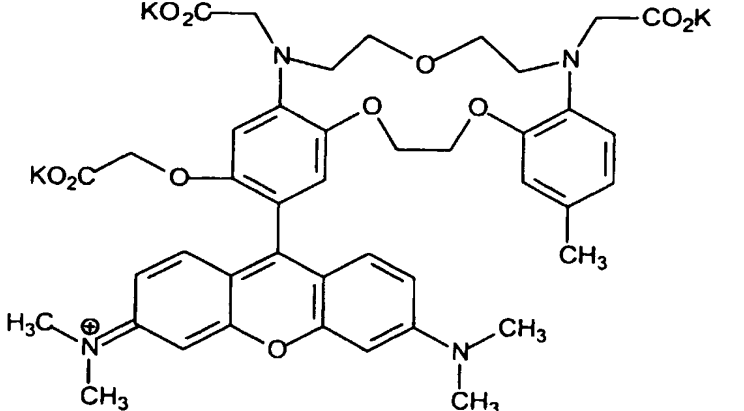
Selected embodiments of the invention are given in Table 1, showing a variety of distinct DYE moieties and crown ether substituents useful in the invention.

Table 1: Selected embodiments of the invention.

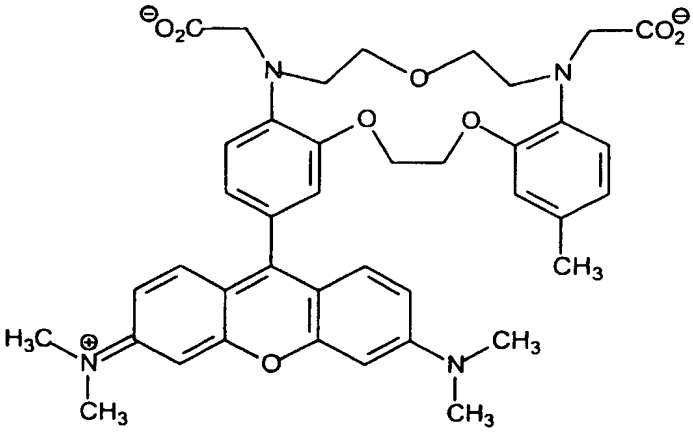
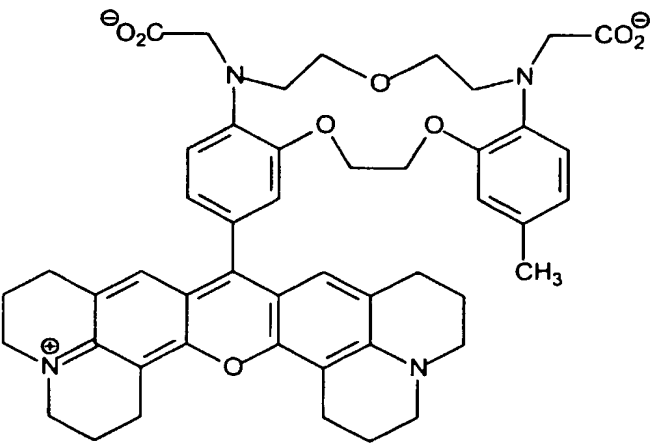
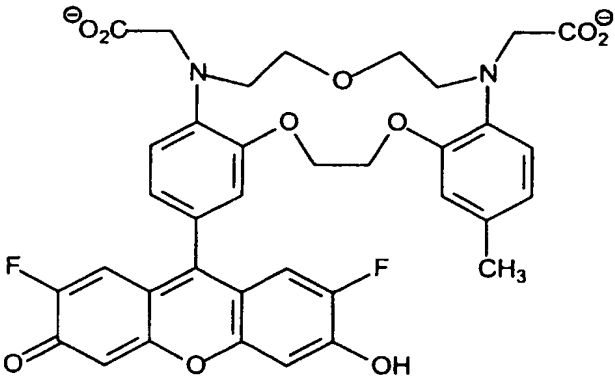
Compound	Dissociation Constant (target ion)
 <p>Chemical structure of Compound 167: A central 1,3-bis(methoxycarbonylmethyl)propan-2-yl ether linker connects two aromatic systems. The left system is a benzofuran derivative with a 2-carboxy-5-methyl-2H-imidazole ring fused to the furan part. The right system is a 4-methylphenyl ring.</p>	$K_d(\text{Na}^+) \sim 52 \text{ mM}$ $K_d(\text{K}^+) \sim 330 \text{ mM}$
 <p>Chemical structure of Compound 148: A central 1,3-bis(methoxycarbonylmethyl)propan-2-yl ether linker connects two aromatic systems. The left system is a 6-methoxycarbonyl-1H-indole-3-carboxamide derivative. The right system is a 4-methylphenyl ring.</p>	$K_d(\text{Na}^+) \sim 30 \text{ mM}$ $K_d(\text{K}^+) \sim 115 \text{ mM}$

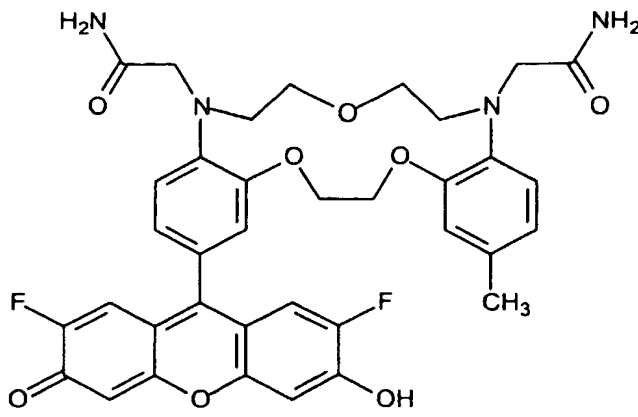
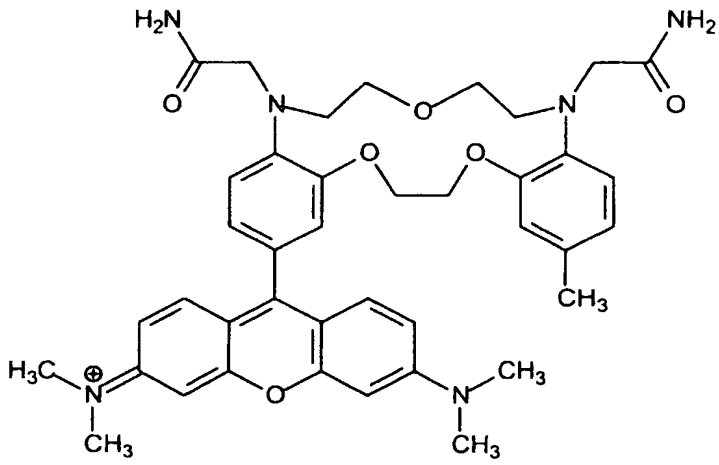
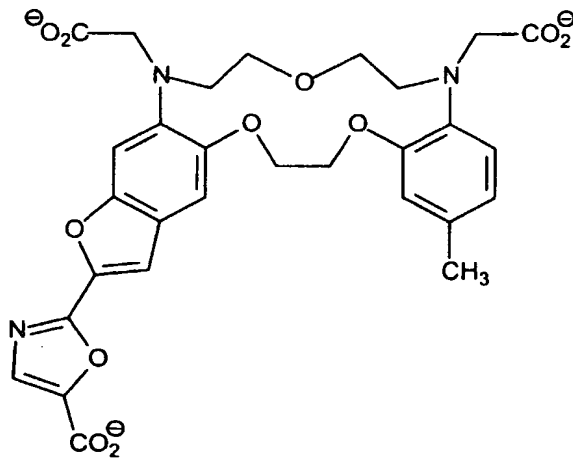
 <p>Compound 180</p>	$K_d (\text{Na}^+) = \sim 220 \text{ mM}$
 <p>Compound 174</p>	$K_d (\text{Na}^+) = \sim 52 \text{ mM}$ $K_d (\text{K}^+) = \sim 250 \text{ mM}$
 <p>Compound 132</p>	$K_d (\text{Na}^+) = \sim 60 \text{ mM}$ $K_d (\text{K}^+) = \sim 205 \text{ mM}$

 <p>Compound 145</p>	<p>$K_d(\text{Na}^+) = \sim 42 \text{ mM}$</p>
 <p>Compound 164</p>	<p>$K_d(\text{Na}^+) = \sim 28 \text{ mM}$ $K_d(\text{K}^+) = \sim 130 \text{ mM}$</p>
	

 <p>Chemical structure of Compound 138: A xanthine derivative with a central pyrimidine ring fused to two benzene rings. The left benzene ring has a dimethylamino group (-N(CH₃)₂) at the 2-position. The right benzene ring has a dimethylamino group (-N(CH₃)₂) at the 6-position. The pyrimidine ring has a methoxy group (-OCH₃) at the 4-position. The pyrimidine ring is connected to a biphenyl system via a methoxy group (-OCH₃) at the 2-position. The biphenyl system has two methoxy groups (-OCH₃) at the 4-positions of the two phenyl rings. The biphenyl system is connected to a dimethylamino group (-N(CH₃)₂) at the 2-position of the right phenyl ring.</p>	<p>K_d (Na⁺) = ~ 95 mM K_d (K⁺) = ~ 300 mM</p>
 <p>Chemical structure of Compound 152: Similar to Compound 138, but the biphenyl system has a methoxy group (-OCH₃) at the 4-position of the left phenyl ring and a methoxy group (-OCH₃) at the 4-position of the right phenyl ring. The biphenyl system is connected to a dimethylamino group (-N(CH₃)₂) at the 2-position of the right phenyl ring.</p>	<p>K_d (Na⁺) = ~ 92 mM K_d (K⁺) = ~ 705 mM</p>
 <p>Chemical structure of Compound 177: Similar to Compound 138, but the biphenyl system has a methoxy group (-OCH₃) at the 4-position of the left phenyl ring and a methoxy group (-OCH₃) at the 4-position of the right phenyl ring. The biphenyl system is connected to a dimethylamino group (-N(CH₃)₂) at the 2-position of the right phenyl ring.</p>	<p>K_d (Na⁺) = ~ 70 mM K_d (Zn²⁺) = ~ 100 nM</p>

	$K_d(\text{Na}^+) = \sim 85 \text{ mM}$ $K_d(\text{K}^+) = \sim 255 \text{ mM}$
<p style="text-align: right;">Compound 156</p>	$K_d(\text{Na}^+) = \sim 14 \text{ mM}$
<p style="text-align: right;">Compound 143</p>	$K_d(\text{Na}^+) = \sim 103 \text{ mM}$ $K_d(\text{K}^+) = \sim 205 \text{ mM}$

 <p>Compound 139</p>	$K_d(\text{Zn}^{2+}) = \sim 300 \text{ nM}$ $K_d(\text{Ca}^{2+}) = \sim 4 \text{ }\mu\text{M}$ $K_d(\text{Na}^+) = \sim 38 \text{ mM}$ $K_d(\text{K}^+) = \sim 270 \text{ mM}$
 <p>Compound 144</p>	$K_d(\text{Zn}^{2+}) = \sim 8 \text{ }\mu\text{M}$ $K_d(\text{Ca}^{2+}) = \sim 7 \text{ }\mu\text{M}$
 <p>Compound 133</p>	$K_d(\text{Na}^+) = \sim 36 \text{ mM}$ $K_d(\text{K}^+) = \sim 215 \text{ mM}$ $K_d(\text{Zn}^{2+}) = \sim 300 \text{ nM}$ $K_d(\text{Ca}^{2+}) = \sim 5 \text{ }\mu\text{M}$

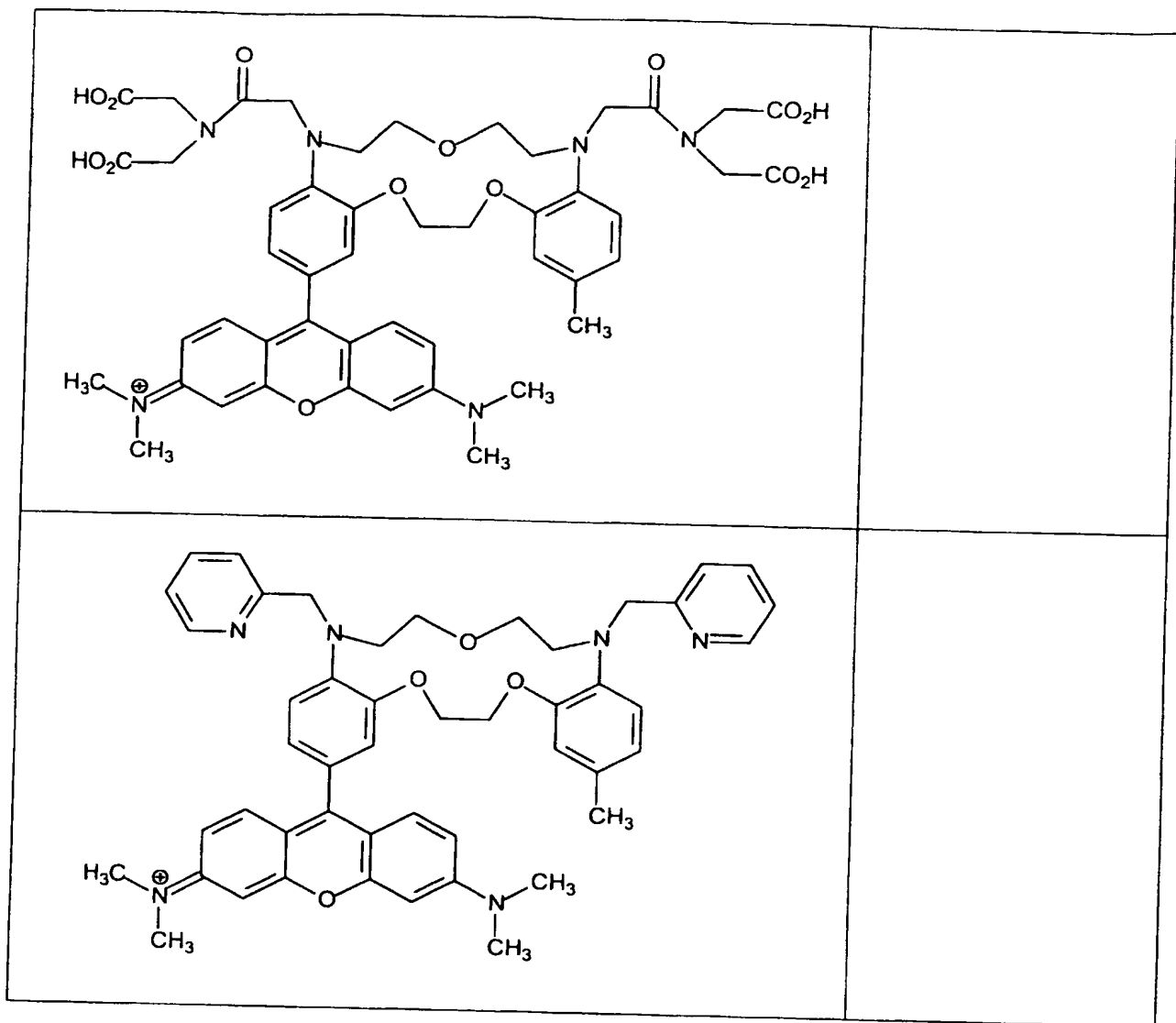
	$K_d (\text{Na}^+) = \sim 2.0 \text{ M}$ $K_d (\text{Zn}^{2+}) = \sim 600 \mu\text{M}$
 <p style="text-align: right;">Compound 182</p>	$K_d (\text{Na}^+) = \sim 500 \text{ mM}$ $K_d (\text{Zn}^{2+}) = \sim 650 \mu\text{M}$
 <p style="text-align: right;">Compound 171</p>	$K_d (\text{Ca}^{2+}) = \sim 400 \text{ nM}$ $K_d (\text{Na}^+) = \sim 160 \text{ mM}$

Reactive Functional Groups and Conjugated Substances

In one embodiment, the compound of the invention is substituted one or more times by an -L-R_x moiety, where R_x is the reactive group that is bound by an additional covalent linkage L, that may be the same or different from the covalent linkage that binds the DYE moiety to the crown ether chelator. In certain embodiments, the covalent linkage contains multiple intervening atoms that serve as a spacer. Compounds incorporating a reactive group (R_x) can be coupled to a wide variety of organic or inorganic substances that contain or are modified to contain functional groups with suitable reactivity, resulting in chemical attachment of the conjugated substance (S_c), represented by -L-S_c. The reactive group and functional group are typically an electrophile and a nucleophile that can generate a covalent linkage. Alternatively, the reactive group is a photoactivatable group, and becomes chemically reactive only after illumination with light of an appropriate wavelength. Typically, the conjugation reaction between the reactive group and the substance to be conjugated results in one or more atoms of the reactive group R_x to be incorporated into a new linkage L attaching the compound of the invention to the conjugated substance S_c. Selected examples of functional groups and linkages are shown in Table 2, where the reaction of an electrophilic group and a nucleophilic group yields a covalent linkage.

Table 2: Examples of some routes to useful covalent linkages

Electrophilic Group	Nucleophilic Group	Resulting Covalent Linkage
activated esters*	amines/anilines	carboxamides
acrylamides	thiols	thioethers
acyl azides**	amines/anilines	carboxamides
acyl halides	amines/anilines	carboxamides
acyl halides	alcohols/phenols	esters
acyl nitriles	alcohols/phenols	esters
acyl nitriles	amines/anilines	carboxamides
aldehydes	amines/anilines	imines
aldehydes or ketones	hydrazines	hydrazones
aldehydes or ketones	hydroxylamines	oximes
alkyl halides	amines/anilines	alkyl amines
alkyl halides	carboxylic acids	esters
alkyl halides	thiols	thioethers
alkyl halides	alcohols/phenols	ethers
alkyl sulfonates	thiols	thioethers
alkyl sulfonates	carboxylic acids	esters
alkyl sulfonates	alcohols/phenols	ethers
anhydrides	alcohols/phenols	esters



anhydrides	amines/anilines	carboxamides
aryl halides	thiols	thiophenols
aryl halides	amines	aryl amines
aziridines	thiols	thioethers
boronates	glycols	boronate esters
carbodiimides	carboxylic acids	N-acylureas or anhydrides
diazoalkanes	carboxylic acids	esters
epoxides	thiols	thioethers
haloacetamides	thiols	thioethers
haloplatinate	amino	platinum complex
haloplatinate	heterocycle	platinum complex
haloplatinate	thiol	platinum complex
halotriazines	amines/anilines	aminotriazines
halotriazines	alcohols/phenols	triazinyl ethers
halotriazines	thiols	triazinyl thioethers
imido esters	amines/anilines	amidines
isocyanates	amines/anilines	ureas
isocyanates	alcohols/phenols	urethanes
isothiocyanates	amines/anilines	thioureas
maleimides	thiols	thioethers
phosphoramidites	alcohols	phosphite esters
silyl halides	alcohols	silyl ethers
sulfonate esters	amines/anilines	alkyl amines
sulfonate esters	thiols	thioethers
sulfonate esters	carboxylic acids	esters
sulfonate esters	alcohols	ethers
sulfonyl halides	amines/anilines	sulfonamides
sulfonyl halides	phenols/alcohols	sulfonate esters

* Activated esters, as understood in the art, generally have the formula $-\text{CO}\square$, where \square is a good leaving group (e.g., succinimidyl $(-\text{OC}_4\text{H}_4\text{O}_2)$ sulfosuccinimidyl $(-\text{OC}_4\text{H}_3\text{O}_2-\text{SO}_3\text{H})$, -1-oxybenzotriazolyl $(-\text{OC}_6\text{H}_4\text{N}_3)$; or an aryloxy group or aryloxy substituted one or more times by electron withdrawing substituents such as nitro, fluoro, chloro, cyano, or trifluoromethyl, or combinations thereof, used to form activated aryl esters; or a carboxylic acid activated by a carbodiimide to form an anhydride or mixed anhydride $-\text{OCOR}^a$ or $-\text{OCNR}^a\text{NHR}^b$, where R^a and R^b , which may be the same or different, are C_1 - C_6 alkyl, C_1 - C_6 perfluoroalkyl, or C_1 - C_6 alkoxy; or cyclohexyl, 3-dimethylaminopropyl, or N-morpholinoethyl).

** Acyl azides can also rearrange to isocyanates

The covalent linkage L binds the reactive group Rx or conjugated substance Sc to the compound of the invention, either directly (L is a single bond) or with a combination of stable chemical bonds, optionally including single, double, triple or aromatic carbon-carbon bonds, as well as carbon-nitrogen bonds.

bonds, carbon-phosphorus bonds, phosphorus-oxygen bonds, phosphorus-nitrogen bonds, nitrogen-platinum bonds, and carbon-silicon bonds. L typically includes ether, thioether, carboxamide, sulfonamide, urea, urethane or hydrazine moieties. In one embodiment, the covalent linkage incorporates a platinum atom, such as described in U.S. Patent No. 5,714,327 (incorporated by reference).

Preferred L moieties have 1-20 nonhydrogen atoms selected from the group consisting of C, N, O, P, and S; and are composed of any combination of ether, thioether, amine, ester, carboxamide, sulfonamide, hydrazide bonds and aromatic or heteroaromatic bonds. Preferably L is a combination of single carbon-carbon bonds and carboxamide or thioether bonds. The longest linear segment of the linkage L preferably contains 4-10 nonhydrogen atoms, including one or two heteroatoms. Examples of L include substituted or unsubstituted polymethylene, arylene, alkylarylene, arylenealkyl, or arylthio.

In one embodiment, L contains 1-6 carbon atoms; in another, L comprises a thioether linkage. In another embodiment, L is or incorporates the formula $-(CH_2)_d(CONH(CH_2)_e)_z-$ or $-O(CH_2)_d(CONH(CH_2)_e)_z-$, where d is an integer from 0-5, e is an integer from 1-5 and z is 0 or 1. In a further embodiment, L is or incorporates the formula $-O-(CH_2)-$. In yet another embodiment, L is or incorporates a phenylene or a 2-carboxy-substituted phenylene.

Choice of the reactive group used to attach the compound of the invention to the substance to be conjugated typically depends on the functional group on the substance to be conjugated and the type or length of covalent linkage desired. The types of functional groups typically present on the organic or inorganic substances include, but are not limited to, amines, amides, thiols, alcohols, phenols, aldehydes, ketones, phosphates, imidazoles, hydrazines, hydroxylamines, disubstituted amines, halides, epoxides, silyl halides, carboxylate esters, sulfonate esters, purines, pyrimidines, carboxylic acids, olefinic bonds, or a combination of these groups. A single type of reactive site may be available on the substance (typical for polysaccharides or silica), or a variety of sites may occur (e.g., amines, thiols, alcohols, phenols), as is typical for proteins. A conjugated substance may be conjugated to more than one dye, which may be the same or different, or to a substance that is additionally modified by a hapten, such as biotin. Although some selectivity can be obtained by careful control of the reaction conditions, selectivity of labeling is best obtained by selection of an appropriate reactive functional group.

Typically, Rx will react with an amine, an alcohol, an aldehyde, a ketone, or with silica. Preferably Rx reacts with an amine or a thiol functional group, or with silica. In one embodiment, Rx is an acrylamide, an activated ester of a carboxylic acid, an acyl azide, an acyl nitrile, an aldehyde, an alkyl halide, a silyl halide, an anhydride, an aniline, an aryl halide, an azide, an aziridine, a boronate, a diazoalkane, a haloacetamide, a halotriazine, a hydrazine

(including hydrazides), an imido ester, an isocyanate, an isothiocyanate, a maleimide, a phosphoramidite, a reactive platinum complex, a sulfonyl halide, or a thiol group. By "reactive platinum complex" is particularly meant chemically reactive platinum complexes such as described in U.S. Patent No. 5,714,327 (incorporated by reference).

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Where Rx is an activated ester of a carboxylic acid, the resulting compound is particularly useful for preparing conjugates of proteins, nucleotides, oligonucleotides, or haptens. Where Rx is a maleimide or haloacetamide the resulting compound is particularly useful for conjugation to thiol-containing substances. Where Rx is a hydrazide, the resulting compound is particularly useful for conjugation to periodate-oxidized carbohydrates and glycoproteins, and in addition is an aldehyde-fixable polar tracer for cell microinjection. Where Rx is a silyl halide, the resulting compound is particularly useful for conjugation to silica surfaces, particularly where the silica surface is incorporated into a fiber optic probe subsequently used for remote ion detection or quantitation.

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Preferably, Rx is a succinimidyl ester of a carboxylic acid, a haloacetamide, a hydrazine, an isothiocyanate, a maleimide group, an aliphatic amine, a silyl halide, or a psoralen. More preferably, Rx is a succinimidyl ester of a carboxylic acid, a maleimide, an iodoacetamide, or a silyl halide. In a particular embodiment Rx is a silyl halide or an isothiocyanate.

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The compounds of the invention that possess a reactive functional group are useful for conjugation to any substance that possesses a suitable functional group for covalent attachment of the chelate. Examples of particularly useful conjugates include, among others, conjugates of antigens, steroids, vitamins, drugs, haptens, metabolites, toxins, environmental pollutants, amino acids, peptides, proteins, nucleic acids, nucleic acid polymers, carbohydrates, lipids, and non-biological polymers. Alternatively, these are conjugates of cells, cellular systems, cellular fragments, or subcellular particles. Examples include, among others, virus particles, bacterial particles, virus components, biological cells (such as animal cells, plant cells, bacteria, yeast, or protists), or cellular components.

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Preferably the conjugated substance is a protein, polysaccharide, lipid, lipid assembly, non-biological polymer, or polymeric microparticle. Another class of preferred conjugated substances includes particles or fibers composed of silica or other glasses, useful for preparing optical devices for remote sensing.

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Preferred nucleic acid polymer conjugates are labeled, single- or multi-stranded, natural or synthetic DNA or RNA, DNA or RNA oligonucleotides, or DNA/RNA hybrids, or incorporate an unusual linker such as morpholine derivatized phosphates (AntiVirals, Inc., Corvallis OR), or peptide nucleic acids such as *N*-(2-aminoethyl)glycine units.

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In a preferred embodiment, the conjugated substance (S_c) is a carbohydrate that is typically a polysaccharide, such as a dextran, FICOLL, heparin, glycogen, amylopectin, mannan, inulin, starch, agarose and cellulose. Alternatively, the carbohydrate is a polysaccharide that is a lipopolysaccharide. Preferred polysaccharide conjugates are dextran, FICOLL, or lipopolysaccharide conjugates.

In another embodiment, the conjugated substance (S_c), is a lipid moiety (typically having 6–60 carbons), including glycolipids, phospholipids, sphingolipids, and steroids. Alternatively, the conjugated substance is a lipid assembly, such as a liposome. The lipid moiety may be used to retain the conjugated substances in cells, as described in U.S. Pat. 5,208,148 (incorporated by reference).

Other conjugates of non-biological materials include conjugates of organic or inorganic polymers, polymeric films, polymeric wafers, polymeric membranes, polymeric particles, or polymeric microparticles, including magnetic and non-magnetic microspheres, conducting and non-conducting metals and non-metals, and glass and plastic surfaces and particles. Conjugates are optionally prepared by copolymerization of a compound of the invention that contains an appropriate functionality while preparing the polymer, or by chemical modification of a polymer that contains functional groups with suitable chemical reactivity. Other types of reactions that are useful for preparing conjugates of polymers include catalyzed polymerizations or copolymerizations of alkenes and reactions of dienes with dienophiles, transesterifications or transaminations. In another embodiment, the conjugated substance is a glass or silica, which may be formed into an optical fiber or other structure.

Conjugates typically result from mixing appropriate reactive dyes and the substance to be conjugated in a suitable solvent in which both are soluble. Labeling of insoluble polymers or silica can be performed in a suspension of the insoluble polymer in a suitable solvent. For those reactive groups that are photoactivated, conjugation requires illumination of the reaction mixture to activate the reactive group.

Synthesis

There are typically three components to the methodology used to prepare the compounds of the invention. The first involves the formation of the crown ether chelate itself, the second involves the appropriate derivatization of the secondary amine nitrogen atoms of the crown ether chelate, and the third involves modification of the crown ether chelate by forming a reactive functional group, covalently attaching a conjugate, or covalently attaching a DYE moiety to form an indicator. Although these synthetic components are typically performed in the order given, they may be carried out in any other suitable sequence. For

example, a portion of the chelate may be derivatized with a fluorescent dye prior to formation of the complete chelate ring.

Where the P and Q moieties are both oxygen, and E¹ is ethylene, the crown ether chelate is typically prepared by acylation of a bis-(2-aminophenoxy)ethane with a bis-(acid chloride), such as diglycolyl chloride, followed by reduction of the resulting bis-amide to the corresponding bis-secondary amine. Selection of the appropriate bis-acid chloride results in the particular desired crown ether (as in Examples 64-69 and 70).

The secondary amine nitrogen atoms present in the crown ether are typically derivatized with an alkylating agent. As the metal binding ability of the resulting crown ether is significantly influenced by the nature of the amine substituents, careful selection of the alkylating agent may be necessary to prepare a reporter for a particular target ion. Where the crown nitrogens are alkylated by methyl bromoacetate, the resulting bis-aza-crown ether is typically selective for sodium ions. If the alkylating agent is 2-picolyll chloride, the resulting crown ether is typically selective for zinc ions. As discussed above, the presence of esters vs. carboxylic acids on the amine nitrogen substituents may influence the relative binding affinity of selected target ions. Selection of an alkylating agent that incorporates a precursor to a reactive functional group is useful for producing chemically reactive compounds of the invention, as well as acting as a useful intermediate for preparing conjugates, as described above. Additionally, an alkylating agent that incorporates a reporter DYE results in a crown ether compound that functions as an indicator for selected target ions.

More typically, the crown ether chelate is derivatized at the benzo ring of the crown ether. As described above, typically a suitable crown ether is prepared, and then bound to a DYE moiety. In one aspect of the invention, an ortho-hydroxy aromatic aldehyde is treated with a chloromethyl heterocycle to yield a fused reporter (as described in Example 46). In another aspect of the invention, derivatization with a DYE moiety is carried out by modifying a crown ether that possesses an aldehyde or ketone functional group.

In one aspect of the invention, the crown ether is substituted by an aldehyde and the fluorophore precursors and the crown ether are combined under anaerobic or non-oxidative conditions (e.g., under nitrogen), and subsequently oxidized using a mild oxidant (e.g., a quinone oxidant, preferably DDQ or chloranil). Where xanthene fluorophore precursors are condensed under anaerobic conditions, the resulting fluorophore is the non-fluorescent dihydro species, which may be utilized without prior oxidation as a sensor for oxidative subenvironments, e.g., in cells.

In yet another aspect of the invention, the crown ether is substituted by a carboxylic acid or by an aldehyde that is converted to the carboxylic acid in the course of synthesis of the

○ crown ether. The chelating moiety is then condensed with the fluorophore precursors to yield the resulting indicator directly.

5 Synthesis of conventional xanthene dyes such as fluoresceins, rhodamines and rhodols typically involve the condensation of two equivalents of resorcinol (for fluoresceins), aminophenol (for rhodamines) or a mixture of a resorcinol and an aminophenol (for rhodols) with a carbonyl-containing moiety such as a phthalic acid derivative or benzaldehyde. In the synthesis of the xanthene indicators of the invention, the desired resorcinol or aminophenol is condensed with the substituted crown ether, yielding either the reduced xanthene (where the crown ether contains an aldehyde) or the oxidized xanthene (where the crown ether contains a carboxylic acid or acyl halide) bound directly to the chelating moiety.

10 An oxidation step is typically required after condensation of a formyl-substituted crown ether with the fluorophore precursors. Optionally, the dihydro condensation product is isolated and subsequently oxidized with air or by standard chemical oxidants, such as chloranil. For some fluorophores, the oxidation reaction is enhanced by acidic reaction conditions. These mild oxidation reaction conditions tolerate a wide variety of substituents on the fluorophore and/or crown ether of the resulting indicators.

20 Unsymmetrical xanthene dyes are typically constructed by statistical methods, using a 1:1 mixture of the desired resorcinols or aminophenols in the condensation reaction, and purifying the desired product from the statistical mix of products using methods known in the art.

25 The synthesis of polyazaindacene dyes, particularly dipyrrometheneboron difluoride dyes, has been well documented (U.S. Patent No. 4,774,339, U.S. Patent No. 5,187,288, U.S. Patent No. 5,248,782, U.S. Patent No. 5,274,113, U.S. Patent No. 5,338,854, and U.S. Patent No. 5,433,896, all incorporated by reference). The procedure typically consists of an acid-catalyzed condensation of a benzaldehyde with a pyrrole that has a hydrogen at the 2-position, followed by *in situ* oxidation of the condensed intermediate by air, oxygen or a chemical oxidant such as 2,3-dichloro-5,6-dicyano-1,4-benzoquinone. The condensation of two appropriately substituted pyrroles, each having a hydrogen at the 2-position, with a formyl-substituted crown ether, followed by *in situ* oxidation of the condensed intermediate and treatment with a boron trifluoride etherate yields the dipyrrometheneboron difluoride indicators of the invention. Alternatively, the indicators are formed via the direct condensation of a carboxyl- or chlorocarbonyl-substituted crown ether with two equivalents of appropriately substituted pyrroles, which may be the same or different, provided each has a hydrogen at the 2-position. The latter procedure does not require oxidation.

40 Post-condensation modifications of both the crown ether and the fluorophore moiety

are typically strictly analogous to known methods of indicator modification. For example, the reduction of nitro substituents to amino groups, the conversion of carboxy substituents to cyano groups, and the preparation of esters of carboxylic acids, including acetoxymethyl esters. Additionally, salts and counterions of the indicators of the invention are readily converted to other salts by treatment with ion-exchange resins, selective precipitation, and basification, as is well-known in the art.

Post-condensation modifications of xanthylium dyes are well known. For instance, the xanthenone portion of the dye can be halogenated by treatment with the appropriate halogenating agent, such as liquid bromine. Xanthenes containing unsaturated fused rings can be hydrogenated to the saturated derivatives.

The reduced and oxidized versions of the xanthene indicators are freely interconvertible by well-known oxidation or reduction reagents, including borohydrides, aluminum hydrides, hydrogen/catalyst, and dithionites. Care must be exercised to select an oxidation or reducing agent that is compatible with the crown ether chelator. A variety of oxidizing agents mediate the oxidation of dihydroxanthenes, including molecular oxygen in the presence or absence of a catalyst, nitric oxide, peroxyxynitrite, dichromate, triphenylcarbenium and chloranil. The dihydroxanthenes are also oxidized electrochemically, or by enzyme action, including horseradish peroxidase in combination with peroxides or by nitric oxide.

Rather than condensing the DYE moiety precursors directly with a substituted crown ether, the preformed DYE moiety may be covalently bound to the crown ether via a conventional cross-linking reaction. A wide variety of chemically reactive or potentially chemically reactive and fluorescent fluorescein, rhodamine, rhodol, benzoxanthenes, dibenzoxanthene and other xanthene oxygen heterocycles that absorb maximally beyond about 490 nm are commercially available as described by Haugland, HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS (7th ed., 1999), as described above, or in other literature references. The nature of the bond that links DYE moiety to the crown ether chelate appears to have an effect on the optical response of the DYE moiety to ion binding, sometimes a significant effect. Acceptability of the linking chemistry can be determined by titration of the resultant indicator with the ion of interest over the target range of response (as described in Example 71).

METHOD OF USE

The crown ether compounds of the invention are useful for any application where it is desirable to complex a target metal ion. Selected crown ether compounds of the invention may be useful as ionophores, that is, they facilitate the transport of selected target ions across cell membranes. Where the crown ether compound is bound to a conjugated substance that is a

polymeric matrix, such as a microparticle, or agarose, the compounds are useful for depleting a sample solution of a selected target ion, particularly where the polymeric matrix is used to pack a chromatography column. Other crown ether compounds (those bound to a DYE moiety) are useful as colorimetric or fluorescent indicators for a selected target ion.

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In order for a particular indicator of the present invention to be useful, it must exhibit a detectable change in spectral properties upon complexation of the desired metal ion (target ion) in the chelating moiety. Preferably the change in spectral properties is a change in fluorescence properties. More preferably, the instant indicators display an intensity increase or decrease in emission energy upon the complexation of the desired target ion.

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The specific indicator used in an assay or experiment is selected based on the desired affinity for the target ion as determined by the expected concentration range in the sample, the desired spectral properties, and the desired selectivity. Initially, the suitability of a material as an indicator of ion concentration is commonly tested by mixing a constant amount of the indicating reagent with a measured amount of the target ion under the expected experimental conditions.

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Preferred indicators display a high selectivity, that is, they show a sufficient rejection of non-target ions. The interference of a non-target ion is tested by a comparable titration of the indicator with that ion. Although preferred target ions for most indicators of the present invention are Na^+ , and K^+ , any ion that yields a detectable change in absorption wavelengths, emission wavelengths, fluorescence lifetimes or other measurable optical property over the concentration range of interest is potentially measured using one of the indicators of this invention. Modifications to the electronic structure of the crown ether or indicator to produce an indicator having the appropriate combination of binding affinity, ion selectivity and spectral response for a wide variety of metal ions.

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In one embodiment of the invention, the target ions for the indicators of the present invention are selected from Li^+ , Na^+ , K^+ , Cs^+ , Ca^{2+} , Zn^{2+} , Mg^{2+} , Rb^+ , Tb^{3+} or Eu^{3+} . In another embodiment of the invention, the target ions are selected from Li^+ , Na^+ , K^+ , Ca^{2+} , Zn^{2+} , and Mg^{2+} . Additional target ions for selected embodiments of the present indicators also include Mn^{2+} , Fe^{2+} , Fe^{3+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Cu^+ , Zn^{2+} , Al^{3+} , Cd^{2+} , Ag^+ , Au^+ , Tl^+ , Pd^{2+} , Hg^{2+} , Hg^+ , Sn^{2+} , Pb^{2+} , Sr^{2+} , Ba^{2+} , Mo^{3+} , Ga^{3+} , In^{3+} , La^{3+} , Eu^{3+} , Tb^{3+} , Dy^{3+} , Ru^{3+} , Sc^{3+} , As^{3+} , Sb^{3+} , Cr^{3+} , Bi^{3+} , Ce^{3+} , Ce^{4+} , Pd^{2+} , Pt^{2+} and Pt^{4+} ions. In yet another embodiment of the invention, the target ions of the instant indicators are Fe^{2+} , Fe^{3+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Cu^+ , Zn^{2+} , Al^{3+} , Cd^{2+} , Hg^{2+} , Pb^{2+} , Ba^{2+} , La^{3+} , Tb^{3+} , Cr^{3+} ions. In yet another embodiment, the target ions are selected from the group consisting of Fe^{3+} , Ni^{2+} , Cu^{2+} , Cu^+ , Hg^{2+} , or Pb^{2+} .

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Th indicator is generally prepared for use as a detection reagent by dissolving the indicator in solution at a concentration that is optimal for detection of the indicator at the expected concentration of the target ion. Modifications that are designed to enhance permeability of the indicator through the membranes of living cells, such as acetoxymethyl esters and acetates, may require the indicator to be predissolved in an organic solvent such as dimethylsulfoxide (DMSO) before addition to a cell suspension, where the indicators then readily enter the cells. Intracellular enzymes cleave the esters to the more polar acids and phenols that are then well retained inside the cells. For applications where permeability of cell-membranes is required, the indicators of the invention are typically substituted by only one fluorophore.

A specific indicator of the present invention is useful for the detection and/or quantification of a desired target ion, when the binding of the target ion in the metal ion-binding moiety of the indicator results in a detectable change in spectral properties. Preferably, the change in spectral properties is a detectable fluorescence response.

A detectable fluorescence response, as used herein, is a change in a fluorescence property of the indicator that is capable of being perceived, either by direct visual observation or instrumentally, and the presence or magnitude of which is a function of the presence of a target metal ion in the test sample. This change in a fluorescence property is typically a change in fluorescence quantum yield, fluorescence polarization, fluorescence lifetime, a shift in excitation or emission wavelength or a combination of these effects. The detectable change in a given spectral property is generally an increase or a decrease. However, spectral changes that result in an enhancement of fluorescence intensity and/or a shift in the wavelength of fluorescence emission or excitation are also useful. The change in fluorescence on ion binding is usually due to conformational or electronic changes in the indicator that may occur in either the excited or ground state of the fluorophore, due to changes in electron density at the ion binding site, due to quenching of fluorescence by the bound target metal ion, or due to any combination of these or other effects.

A preferred indicator for a specific target ion is an indicator that shows at least a two-fold change in net fluorescence emission intensity (either higher or lower), or a 1 nanosecond difference in fluorescence lifetime (either shorter or longer), preferably a five-fold or greater change in net fluorescence emission intensity or a 100% change in fluorescence lifetime in response to the target ion. Alternatively, an indicator that exhibits a shift in excitation or emission wavelength of at least 10 nm (either to shorter or longer wavelength) is also preferred, more preferably exhibiting a shift of 25 nm or greater.

The response of an individual indicator to a specific metal ion is dependent on the properties of indicator in the presence and absence of the target ion. the relative electron

densities of the dye and metal binding site and the ability of metal ions to quench fluorescence emission when in close proximity to a fluorophore (heavy atom quenching). In one embodiment of the invention, the indicator is essentially nonfluorescent or has low fluorescence in target ion-free solution and exhibits an increase in fluorescence intensity or
5 fluorescence lifetime (or both) upon target metal ion binding. In yet another embodiment of the invention, the fluorescence intensity remains approximately the same but there is a shift in the excitation or emission spectrum, or both, upon metal ion binding.

The optical response of the indicating reagent is determined by changes in absorbance
10 or fluorescence, preferably fluorescence. If absorbance measurements are used to determine ion concentrations, then it is usually optimal to adjust the optical density of the indicator in the sample over the range of analyte concentration to a value of approximately 0.02 to 2.5 (most preferably 0.1 to 1). For fluorescence measurements, the concentration of the indicator will depend mostly on the sensitivity of the equipment used for its detection.

If the optical response of the indicator will be determined using fluorescence
15 measurements, samples are typically stained with indicator concentrations of 10^{-9} M to 10^{-2} M. The most useful range of analyte concentration is about one log unit above and below the dissociation constant of the ion-indicator complex. This dissociation constant is determined by
20 titration of the indicator with a known concentrations of the target ion, usually over the range of virtually zero concentration to approximately 100 millimolar of the target ion, depending on which ion is to be measured and which indicator is being used. The dissociation constant may be affected by the presence of other ions, particularly ions that have similar ionic radii and charge. It may also be affected by other conditions such as ionic strength, pH, temperature,
25 viscosity, presence of organic solvents and incorporation of the sensor in a membrane or polymeric matrix, or conjugation or binding of the sensor to a protein or other biological molecule. Any or all of these effects need to be taken into account when calibrating an indicator.

The indicator is combined with a sample in a way that will facilitate detection of the
30 target ion concentration in the sample. The sample is generally a fluid or liquid suspension that is known or suspected to contain the target ion. Representative samples include intracellular fluids such as in blood cells, cultured cells, muscle tissue, neurons and the like; extracellular fluids in areas immediately outside of cells; in vesicles; in vascular tissue of
35 plants and animals; in biological fluids such as blood, saliva, and urine; in biological fermentation media; in environmental samples such as water, soil, waste water and sea water; in industrial samples such as pharmaceuticals, foodstuffs and beverages; and in chemical reactors. Detection and quantitation of the target ion in a sample can help characterize the identity of an unknown sample, or facilitate quality control of a sample of known origin.
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C

In one embodiment of the invention, the sample contains cells, and the indicator is combined with the sample in such a way that the indicator is present within the sample cells. By selection of the appropriate chelating moiety, fluorophore, and the substituents thereon, indicators are prepared that will selectively localize in desired organelles, and provide
5 measurements of the target ion in those organelles. Conjugates of the indicators of the invention with organelle-targeting peptides are used to localize the indicator to the selected organelle, facilitating measurement of target ion presence or concentration within the organelle (as described in U.S. Patent No. 5,773,227 to Kuhn et al., (1998), incorporated by reference). Alternatively, selection of a lipophilic fluorophore, or a fluorophore having
10 predominantly lipophilic substituents will result in localization in lipophilic environments in the cell, such as cell membranes. Selection of cationic indicators will typically result in localization of the indicator in mitochondria.

In another aspect of the invention, a composition of matter comprises any of the
15 compounds described above, and optionally includes a metal ion. In one embodiment, the compounds of the invention, in any of the embodiments described above, are associated, either covalently or noncovalently, with a surface such as a microfluidic chip, a silicon chip, a microscope slide, a microplate well, or another solid matrix, and is combined with the sample of interest as it flows over the surface. The detectable optical response is therefore detected on
20 the matrix surface itself, typically by use of an instrumental. This embodiment of the invention is particularly suited to high-throughput screening using automated methods.

Quantification of target ion levels in samples is typically accomplished using the indicators of the present invention by methods known in the art (Haugland, HANDBOOK OF
25 FLUORESCENT PROBES AND RESEARCH CHEMICALS, 1996, pp., 503-544). For example, the ratiometric measurement of ion concentration provides accurate measurement of ion concentrations by the treatment of the fluorescence data as the ratio of excitation or fluorescence intensities at two wavelengths, rather than the absolute intensity at a single wavelength. Using the ratio method, a number of variables that may perturb the ion
30 concentration measurements are eliminated. In particular, ion-dependent factors that affect the signal intensity, such as nonuniform intracellular dye concentrations, probe leakage, dye bleaching and cell thickness, are canceled in the ratio measurements, since these parameters have a similar effect on intensities at both wavelengths. While the ratio method can be used to determine concentrations using observation of either the excitation spectra of the indicator,
35 the emission spectra of the indicator, or both, in the case of the indicators of the present invention, the shift in excitation energy upon binding metal ions makes observation of the excitation spectrum a more useful technique. In either case, to achieve maximal utility, the indicator must be calibrated (to compensate for variance in the dissociation constant of the indicator due to ionic strength, viscosity, or other conditions within the sample). To calibrate
40 the indicator, ionophores such as A-23187, gramicidin, valinomycin, or ionomycin are used.

Non-ratiometric analysis can also be accomplished by calibration with a second fluorescent dye present in the sample.

5 The optical response of the indicator to the ion can be detected by various means that include measuring absorbance or fluorescence changes with an instrument, visually, or by use of a fluorescence sensing device. Several examples of fluorescence sensing devices are known, such as fluorometers, fluorescence microscopes, laser scanners, flow cytometers, and microfluidic devices, as well as by cameras and other imaging equipment. These measurements may be made remotely by incorporation of the fluorescent ion sensor as part of a fiber optic probe. The indicator is covalently
10 attached to the fiber optic probe material, typically glass or functionalized glass (e.g., aminopropyl glass) or the indicator is attached to the fiber optic probe via an intermediate polymer, such as polyacrylamide. The indicator solution is alternatively incorporated non-covalently within a fiber optic probe, as long as there is a means whereby the target ion can come into contact with the indicator solution.

15

Kits

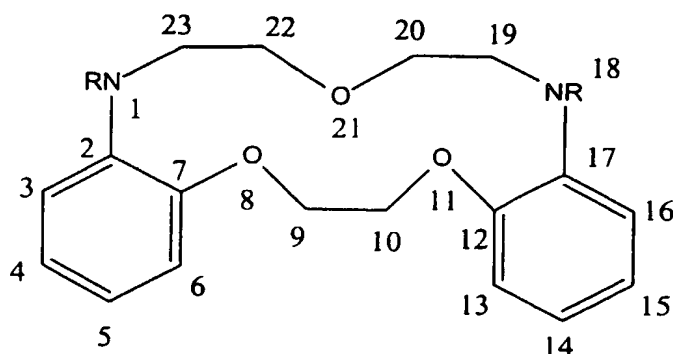
Due to the advantageous properties and the simplicity of use of the instant crown ether compounds, they are particularly useful in the formulation of a kit for the complexation, detection,
20 or quantification of selected target ions, comprising one or more compounds or compositions of the invention in any of the embodiments described above (optionally in a stock solution), instructions for the use of the crown ether compound to complex or detect a desired target ion, and optionally comprising additional components. In one aspect, the compounds of the invention are associated with a surface, such as a chip, microplate well, or other solid matrix, and the sample of interest
25 flows over the surface. The detectable optical response is therefore detected on the matrix surface itself.

The additional kit components may be selected from, without limitation, calibration standards of a target ion, ionophores, fluorescence standards, aqueous buffers, and organic
30 solvents. The additional kit components are present as pure compositions, or as aqueous solutions that incorporate one or more additional kit components. Any or all of the kit components optionally further comprise buffers.

The examples below are given so as to illustrate the practice of this invention. They are not
35 intended to limit or define the entire scope of this invention. Compounds 6, 102, 104-108, 200, 205 and 206 are intermediates.

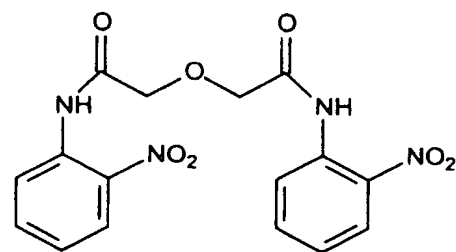
EXAMPLES

40 The following numbering scheme is used throughout the following examples:



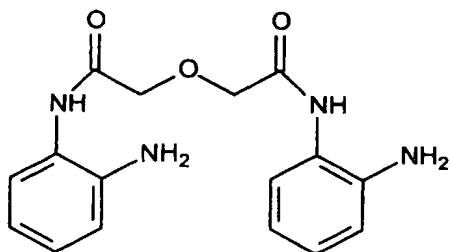
Example 1. Preparation of a tetraaza-crown ether.

2-Nitroaniline is acylated with diglycolyl chloride in anhydrous THF, using 0.5 equivalents of diglycolyl chloride in dilute solution. A 0.1 M THF solution of diglycolyl chloride is slowly added to a 0.1 M THF solution of 2-nitroaniline, containing two equivalents of triethylamine. After TLC indicates consumption of all starting 2-nitroaniline, volatiles are removed in vacuo and the residue is partitioned between water and ethyl acetate. The ethyl acetate layer is concentrated to give Compound 1.



Compound 1

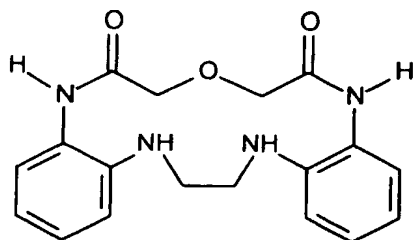
Compound 1 is dissolved in methanol and treated with 20% by weight of 10% palladium on carbon. The resulting mixture is shaken under 40 psi hydrogen until analysis by thin layer chromatography (TLC) indicates the reaction is complete. The reaction mixture is filtered and the bis-aniline 2 is isolated by concentration *in vacuo* of the filtrate.



Compound 2

Compound 2 is dissolved in DMF to achieve a 0.1 M solution, then treated with 0.9 equivalents of 1,2-dibromoethane, two equivalents of diisopropylethylamine, and catalytic sodium iodide. The resulting solution is heated until most of 2 is consumed, as judged by TLC analysis. The

volatiles are removed *in vacuo*, and the residue is partitioned between ethyl acetate and water. The ethyl acetate layer is concentrated *in vacuo* to give compound 3.

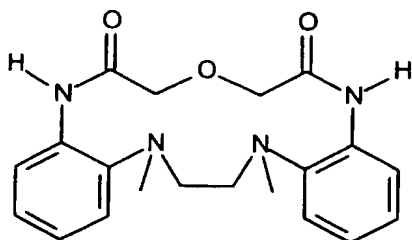


Compound 3

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Compound 3 is dissolved in acetonitrile to achieve a 0.5 M concentration. Ten equivalents of formaldehyde, as a 37% aqueous solution, are added followed by 4 equivalents of sodium cyanoborohydride. The pH of the reaction mixture is adjusted to 7 with acetic acid, and the resulting solution is stirred until TLC analysis indicates consumption of 3 and formation of 4.

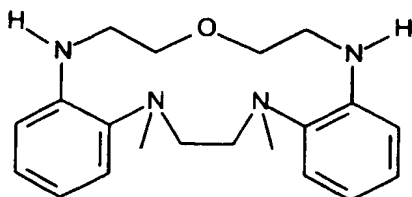
10 Volatiles are removed *in vacuo*, and the residue is partitioned between ethyl acetate and water. The ethyl acetate layer is concentrated *in vacuo* to give compound 4.



Compound 4

15 Compound 4 is dissolved in THF at 1 M concentration, and treated with 10 equivalents of borane-THF (1M). The resulting solution is heated at reflux until 4 is consumed and 5 is formed, as judged by TLC analysis. The reaction is quenched by addition of 5% aqueous sodium hydroxide, followed by extraction with ethyl acetate. The ethyl acetate layer is concentrated *in vacuo* to give 5.

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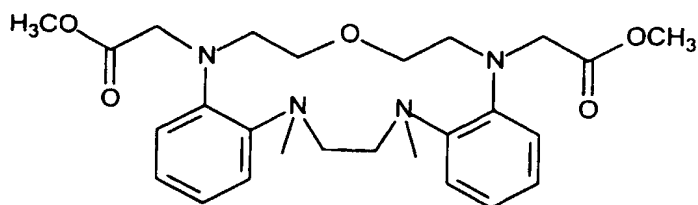


Compound 5

Compound 5 is dissolved in DMF at 0.5 M concentration. Ten equivalents of methyl bromoacetate and 4 equivalents of diisopropylethylamine are added, and the resulting solution is stirred at 100 degrees centigrade until TLC analysis indicates consumption of 5 and formation of 6. Volatiles are removed *in vacuo*, and the residue is partitioned between water

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and ethyl acetate. The ethyl acetate layer is concentrated in vacuo to give 6, which can be further purified by flash chromatography on silica gel using methanol in chloroform as eluant.



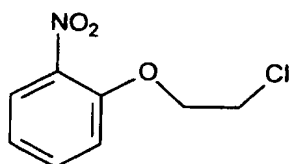
Compound 6

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Example 2. Preparation of (2'-Nitrophenoxy)-2-chloroethane (102).

A suspension of 2-nitrophenol 101 (50.0 g, 0.36 mol), 1-bromo-2-chloroethane (45 mL, 0.54 mol), and K_2CO_3 (100.0 g, 0.72 mol) in DMF (200 mL) was stirred at 90°C for 2 h, cooled to room temperature, poured into ice-water, filtered, washed with H_2O and dried to give Compound 102, 70.3 g (96%) as a yellow solid.

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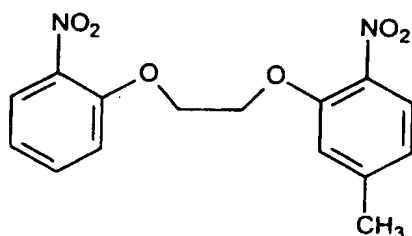


Compound 102

Example 3. Preparation of 1-(5'-methyl-2'-nitrophenoxy)-2-(2''-nitrophenoxy)ethane (104).

A suspension of Compound 102 (60.45 g, 0.30 mol), 5-methyl-2-nitrophenol (50.49 g, 0.33 mol), and K_2CO_3 (82.80 g, 0.60 mol) in DMF (450 mL) was stirred at 130 °C for 18 h, cooled to room temperature, poured into ice-water, filtered, washed with H_2O and dried to give Compound 104, 89.50 g (93.5%) as an orange solid.

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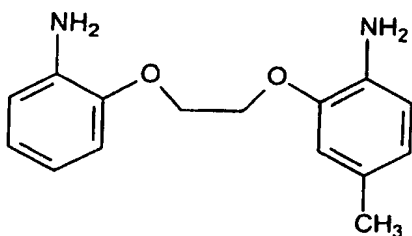
Compound 104

Example 4. Preparation of 1-(2'-amino-5'-methylphenoxy)-2-(2''-aminophenoxy)ethane (105).

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Compound 104 (24.0 g, 75 mmol) was hydrogenated over 10% Pd/C (1.5 g) in DMF (250 mL) at 40 psi for 18 h. The mixture was filtered from catalyst through a CELITE ^(Rm) pad on a fritted

glass filter. The filtrate was evaporated and ether (50 mL) was added. The product was filtered, washed with ether and dried to give compound **105**, 18.0 g (95%) as a brown solid.



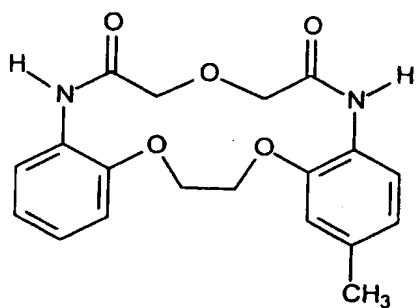
Compound **105**

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Example 5. Preparation of 5-methyl-19,23-dioxo-DDTCPD (106).

To a stirred mixture of diamine **105** (13.10 g, 50 mmol) and Et₃N (20.8 mL, 150 mmol) in dry THF (2.5 L) was slowly added over 12 h a solution of diglycolyl dichloride (6.3 mL, 55 mmol) in dry THF (0.5 L). The reaction mixture was stirred for 6 h, filtered from the precipitated hydrochloride, and washed with THF. The combined organic filtrate was evaporated. The residue was dissolved in CHCl₃ (800 mL) and washed successively with 0.5 M HCl, H₂O, saturated NaHCO₃ then saturated NaCl. The organic layer was dried over MgSO₄ and evaporated. Ether (100 mL) was added and the precipitated product was filtered, washed with ether and dried to give Compound **106**, 16.0 g (90%) as an off-white solid.

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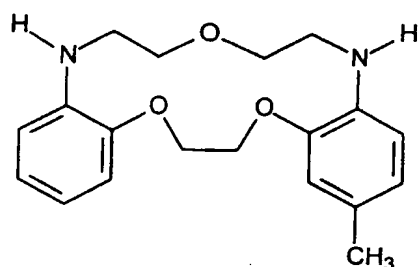
Compound **106**

Example 6. Preparation of 5-methyl-DDTCPD (107).

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To a suspension of diamide **106** (15.60 g, 44 mmol) in dry THF (400 mL) was added a 1 M solution of BH₃-THF complex in dry THF (390 mL, 390 mmol). The mixture was stirred at 70 °C for 16 h. Dry methanol (300 mL) was added dropwise over 1 h into a boiling mixture with strong gas evolution. The mixture was heated at reflux for 2 h, cooled to room temperature, evaporated and co-evaporated with MeOH to destroy the borane complex. Ether (100 mL) was added. The solid product was filtered, washed with ether and dried to give Compound **107**, 12.3 g (85%) as an off-white solid.

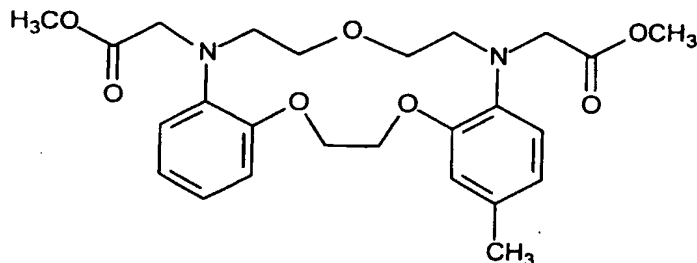
25



Compound 107

Example 7. Preparation of 1,18-bis(methoxycarbonylmethyl)-5-methyl-DDTCPD (108).

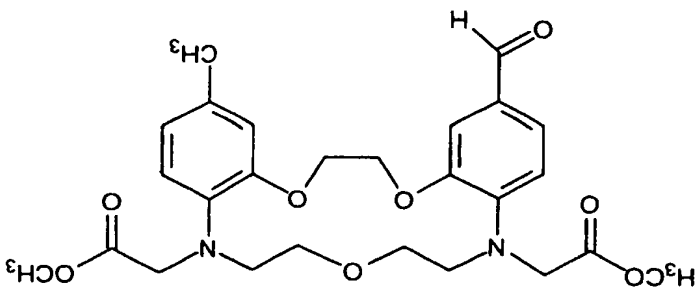
- 5 A mixture of diamine 107 (6.56 g, 20 mmol), methyl bromoacetate (38 mL, 400 mmol), diisopropylethylamine (DIPEA) (104 mL, 600 mmol), and NaI (1.50 g, 10 mmol) in MeCN (300 mL) was heated at reflux for 70 h. After cooling to room temperature, the MeCN was evaporated. The residue was dissolved in CHCl₃, washed with 1% AcOH then H₂O, dried and evaporated. The residual oily product was cooled to 0 °C and washed with cold hexane to
- 10 remove most of the alkylating reagent. The crude product 108 was used immediately in the next step without further purification.



Compound 108

15 Example 8. Preparation of 1,18-bis(methoxycarbonylmethyl)-5-formyl-14-methyl-DDTCPD (109).

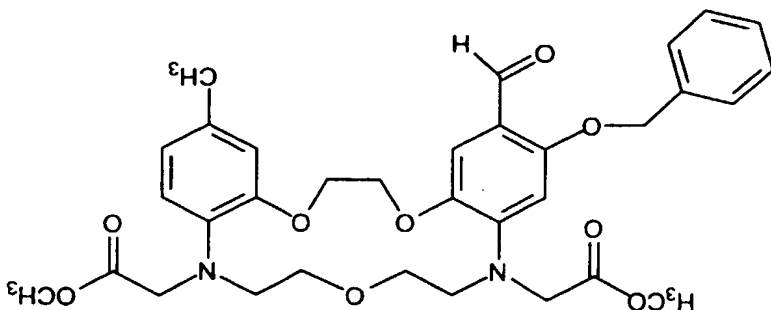
- To a stirred solution of the Vilsmeier reagent prepared from POCl₃ (4.65 mL, 50 mmol) and 30 mL DMF was added over 5 min a solution of Compound 108 (4.8 g, 10 mmol) in DMF (20 mL).
- 20 The mixture was stirred for 16 h, cooled by the addition of ice and neutralized with saturated K₂CO₃ to pH 7-8. The suspension was extracted with CHCl₃, washed successively with 0.1 M HCl, saturated NaCl, saturated NaHCO₃ and saturated NaCl, then evaporated. The residue was dissolved in CHCl₃ and chromatographed on SiO₂ with an EtOAc gradient in hexanes (20% to 30%) to give aldehyde 109, 2.60 g (52%) as colorless crystals.



Compound 109

Example 9. Preparation of 1,18-bis(methoxycarbonylmethyl)-4-benzyl-2-nitrophenyl-
DDTCPD (117):

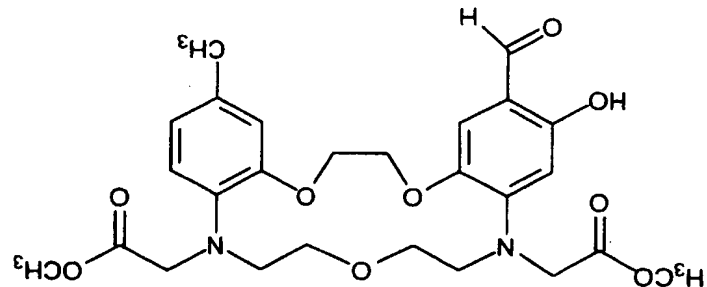
Compound 117 was prepared analogously with Compound 109, starting with 5-methyl-2-nitrophenol in place of 2-nitrophenol, and utilizing 4-benzyl-2-nitrophenol in place of 5-methyl-2-nitrophenol.



Compound 117

Example 10. Preparation of 1,18-bis(methoxycarbonylmethyl)-5-formyl-4-hydroxy-14-methyl-
DDTCPD (118):

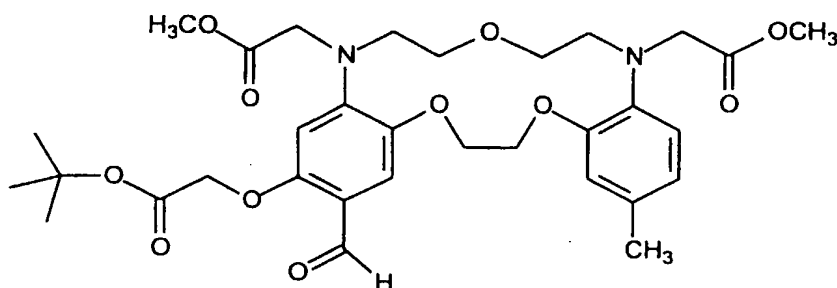
Compound 117 (3.06 g, 5.0 mmol) was hydrogenated over 10% Pd/C (0.50 g) in AcOH (100 mL) at 50 psi for 5 h. The mixture was filtered through a CELTITE pad on a fritted glass filter. The filtrate was evaporated, ether (50 mL) was added, the product was filtered, washed with ether and dried to give Compound 118, 2.13 g (82%) as a colorless solid.



Compound 118

Example 11. Preparation of 1,18-bis(methoxycarbonylmethyl)-4-*t*-butoxycarbonylmethoxy-5-formyl-14-methyl-DDTCPD (119).

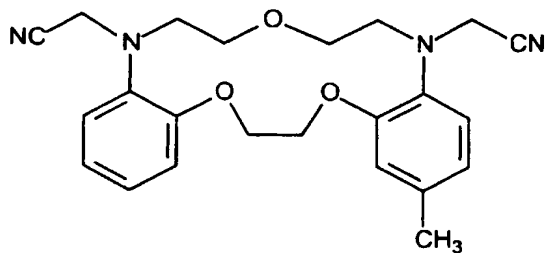
To a suspension of Compound 118 (0.800 g, 1.55 mmol), K₂CO₃ (1.04 g, 7.5 mmol) and NaI (0.03 g, 0.2 mmol, catalyst) in DMF (10 mL) was added dropwise *t*-butyl bromoacetate (0.45 mL, 3.0 mmol). The mixture was stirred for 16 h, diluted with H₂O, extracted with CHCl₃, dried over MgSO₄ and evaporated. The residue was dissolved in hexane: EtOAc (1:1) and chromatographed on silica gel with 40% EtOAc in hexanes to give aldehyde 119, 0.875 g (90%) as colorless crystals.



Compound 119

Example 12. Preparation of 1,18-bis(cyanomethyl)-5-methyl-DDTCPD (120).

A stirred mixture of diamine 107 (3.38 g, 10 mmol), bromoacetonitrile (14 mL, 200 mmol), DIPEA (26 mL, 150 mmol), NaI (1.5 g, 10 mmol; catalyst) in MeCN (150 mL) was heated at reflux for 70 h. After cooling to room temperature the MeCN was evaporated. The residue was dissolved in CHCl₃ (800 mL), washed with H₂O, dried over MgSO₄ and evaporated. The residue was chromatographed on SiO₂ with 40% EtOAc in hexanes to give Compound 120, 3.56 g (90%) as a colorless solid.

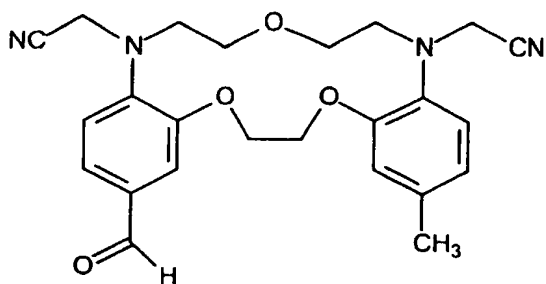


Compound 120

Example 13. Preparation of 1,18-bis(cyanomethyl)-5-formyl-14-methyl-DDTCPD (121).

To a stirred solution of the Vilsmeier reagent prepared from POCl₃ (9.5 mL, 102 mmol) and 60 mL DMF was added over 5 min to a solution of Compound 120. The mixture was stirred for 100 h at 40 °C, cooled by the addition of ice and neutralized with saturated K₂CO₃ to pH 8. The suspension was extracted with CHCl₃, washed with H₂O, dried over MgSO₄ and evaporated.

evaporated. The residue was chromatographed over SiO₂ using CHCl₃ as eluant to give compound 121, 3.12 g (73%) as colorless crystals.



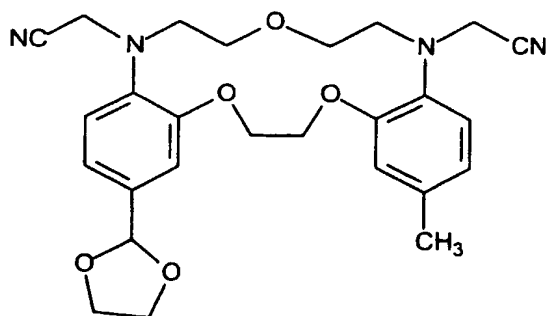
Compound 121

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Example 14. Preparation of 1,18-bis(cyanomethyl)-5-(2,5'-dioxacyclopentyl)-14-methyl-DDTCPD (122).

A mixture of aldehyde 121 (3.08 g, 7.3 mmol), ethylene glycol (8 mL, 140 mmol), p-toluenesulphonic acid (0.20 g, catalyst) and benzene (80 mL) was refluxed with a Dean-Stark trap for 4 h. After cooling to room temperature the mixture was evaporated. The residue was dissolved in CHCl₃ (300 mL), washed with saturated NaHCO₃, dried over MgSO₄, and evaporated to give compound 122, 2.82 g (85%) as an orange solid. Compound 122 was pure on TLC and used in the next step without additional purification.

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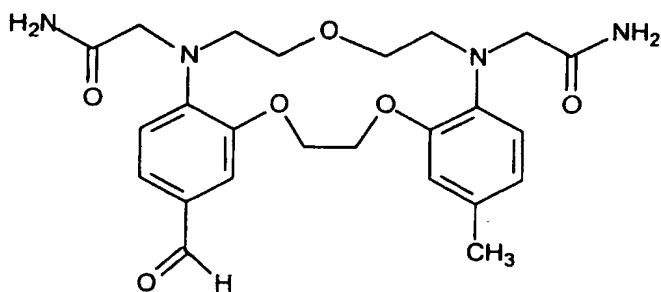


Compound 122

Example 15. Preparation of 1,18-bis(carbamoylmethyl)-5-formyl-14-methyl-DDTCPD (123).

To a solution of Compound 122 (0.200 g, 0.44 mmol) in MeOH (25 mL) and 1 M NaOH (15 mL, 15 mmol) was added over 5 min 30% H₂O₂ (5 mL, catalyst). The mixture was stirred for 1 h, then acidified with 1 M HCl to pH 1. The acidified mixture was stirred for 30 min, diluted with 3 M NaOAc (150 mL), extracted with CHCl₃, washed with saturated NaHCO₃, dried over MgSO₄ and evaporated to give aldehyd 123, 0.191 g (95%) as a colorless crystalline material, pure by TLC.

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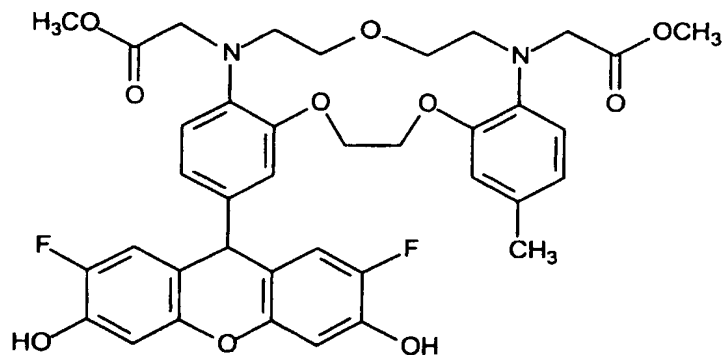
Compound 123

Example 16. Preparation of 1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[2H-fluoro]-DDTCPD (131).

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A mixture of aldehyde **109** (1.150 g, 2.3 mmol) and 4-fluororesorcinol (0.670 g, 5.2 mmol) in MeSO₃H (25 mL) was stirred overnight, then poured into 3 M NaOAc (300 mL). The precipitated solid was filtered, washed with H₂O and dried to give compound **131**, 1.605 g (97%) as an off-white solid. Compound **131** was unstable to oxidation and was used in the next step without additional purification.

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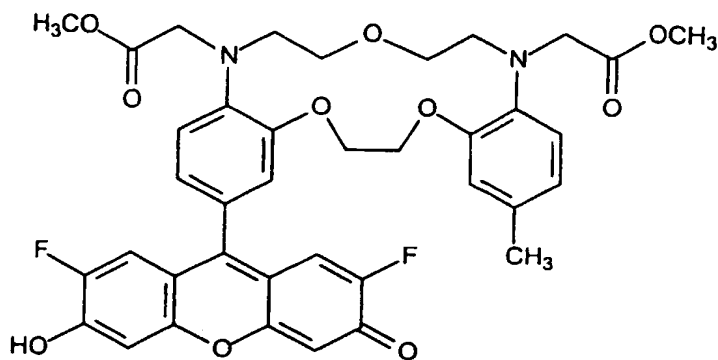
Compound 131

Example 17. Preparation of 1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[fluoro]-DDTCPD (132).

15

A mixture of Compound **131** (150 mg, 0.21 mmol) and chloranil (246 mg, 1.0 mmol) in MeOH (5 mL) and CHCl₃ (5 mL) was refluxed for 50 h, then cooled to room temperature, filtered from excess oxidizer, and evaporated. The residue was purified by preparative TLC on two SiO₂ plates using CHCl₃: MeOH:AcOH (20:2:1) to give compound **132**, 44 mg (20%) as a red-brown solid.

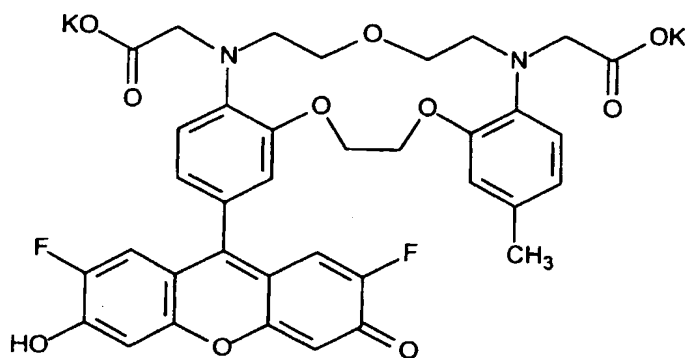
20



Compound 132

Example 18. Preparation of 1,18-bis(carboxymethyl)-14-methyl-5-[fluoro]-DDTCPD (133).

- 5 A mixture of Compound 132 (30 mg, 0.04 mmol) in MeOH (5 mL) and 1 M KOH (1 mL, 1.0 mmol) was stirred for 16 h, then neutralized with 1 M HCl to pH 7, and evaporated to dryness. The residue was purified by preparative TLC on two SiO₂ plates using CHCl₃: MeOH:AcOH (13:3:1) to give compound 133, 21 mg (74%) as a brown solid.



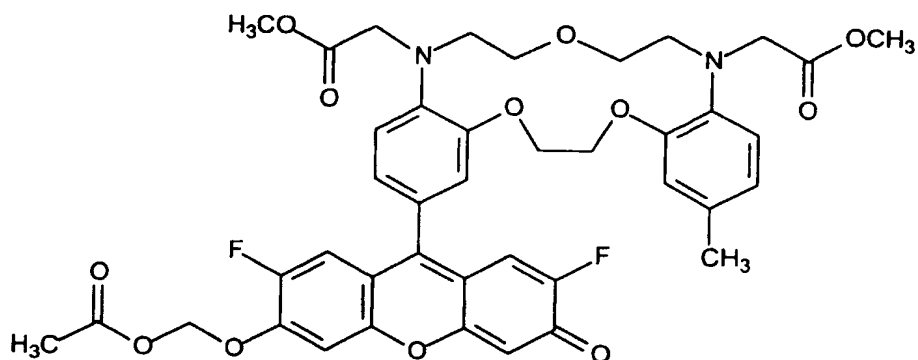
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Compound 133

Example 19. Preparation of 1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[fluoro-AM]-DDTCPD (134).

- 15 A mixture of Compound 132 (78 mg, 0.1 mmol), bromomethyl acetate (0.05 mL, 0.5 mmol), and DIPEA (0.175 mL, 1 mmol) in DMF (1 mL) was stirred for 2 h, then poured into 1% AcOH (200 mL). The suspension was extracted with CHCl₃, washed with H₂O, filtered and evaporated. The residue was purified by preparative TLC on two SiO₂ plates using 5% MeOH in CHCl₃ to give compound 134, 25 mg (32%) as a brown solid.

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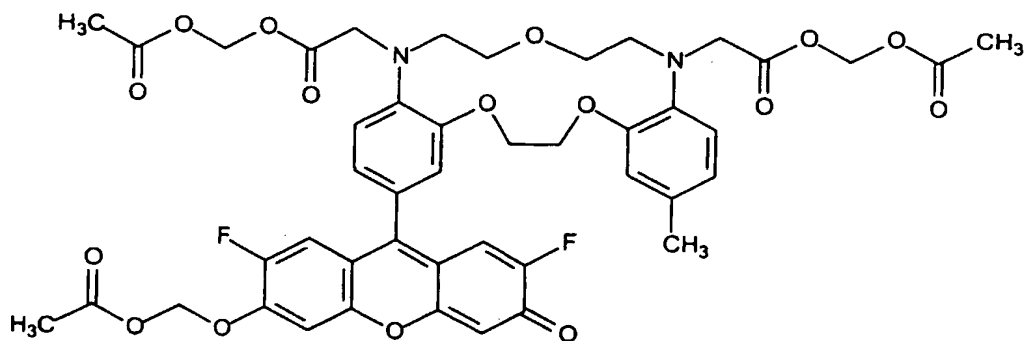
Compound 134

Example 20. Preparation of 1,18-bis(acetoxymethoxycarbonylmethyl)-14-methyl-5-[fluoro-AM]-DDTCPPD (135).

5

A mixture of Compound 133 (69 mg, 0.1 mmol), bromomethyl acetate (0.05 mL, 0.5 mmol), and DIPEA (0.175 mL, 1 mmol) in DMF (1 mL) was stirred for 2 h, then poured into 1% AcOH (200 mL). The suspension was extracted with CHCl_3 , washed with H_2O , filtered and evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 5% MeOH in CHCl_3 to give Compound 135 as a brown solid.

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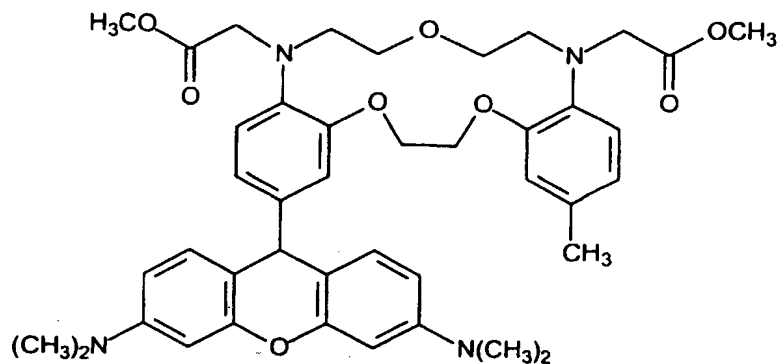
Compound 135

Example 21. Preparation of 1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[2H-TMR]-DDTCPPD (137).

15

A mixture of aldehyde 109 (250 mg, 0.5 mmol), 3-dimethylaminophenol (157 mg, 1.1 mmol), and p-toluenesulphonic acid (10 mg, catalyst) in propionic acid (5 mL) was stirred overnight at 60 °C, then cooled to room temperature and poured into 3 M NaOAc (100 mL). The precipitated solid was filtered, washed with H_2O and dried to give compound 137, 360 mg (97%) as a rose-colored solid. Compound 137 was unstable to oxidation and was used in subsequent reactions without additional purification.

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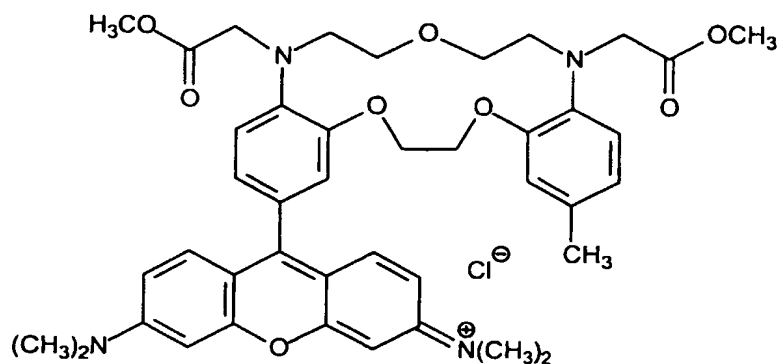
Compound 137

Example 22. Preparation of 1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (138).

5

A mixture of Compound 137 (660 mg, 0.81 mmol) and chloranil (400 mg, 1.62 mmol) in MeOH (25 mL) and CHCl₃ (25 mL) was stirred for 2 h, filtered from excess oxidizer, and evaporated. The residue was purified by chromatography on SiO₂ using 9% MeOH and 1% AcOH in CHCl₃ as eluant to give the crude product, which was again chromatographed on SiO₂ using the same eluant to give Compound 138, 112 mg (19%) as crimson solid.

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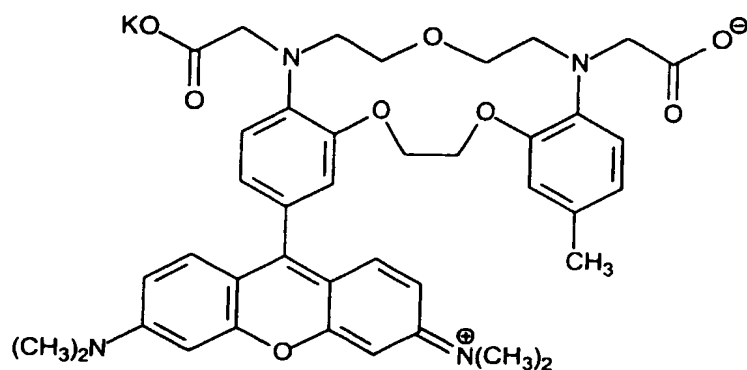
Compound 138

Example 23. Preparation of 1,18-bis(carboxymethyl)-14-methyl-5-[TMR]-DDTCPD potassium salt (139).

15

A mixture of Compound 138 (40 mg, 0.05 mmol) in MeOH (2 mL) and 1 M KOH (1 mL, 1.0 mmol) was stirred for 16 h, then evaporated to dryness. The residue was purified by chromatography on a SEPHADEX/LH-20 column using H₂O as eluant to give salt 139, 8 mg (21%) as red flakes after lyophilization.

20



Compound 139

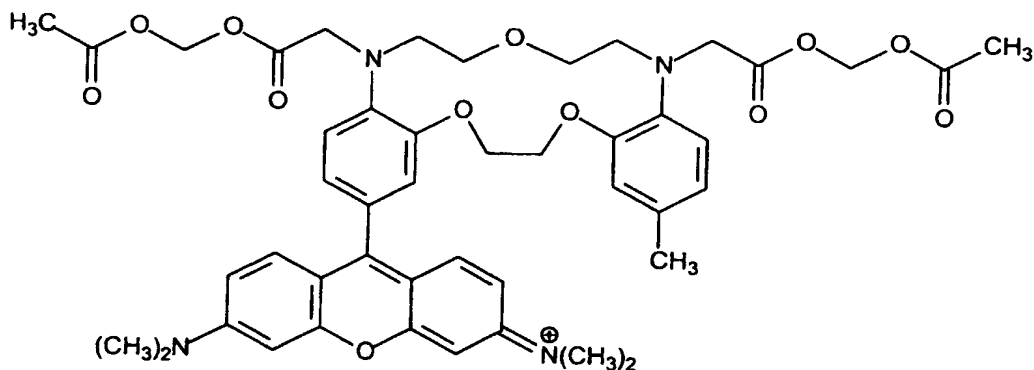
Example 24. Preparation of 1,18-bis(acetoxymethoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (140).

5

A mixture of Compound 139 (75 mg, 0.1 mmol), bromomethyl acetate (0.1 mL, 1.0 mmol), and DIPEA (0.35 mL, 2 mmol) in DMF (2 mL) was stirred for 2 h, then poured into 1% AcOH (200 mL). The suspension is extracted with CHCl_3 , washed with H_2O , filtered and evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 5% MeOH in CHCl_3 to give

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Compound 140 as a red solid.



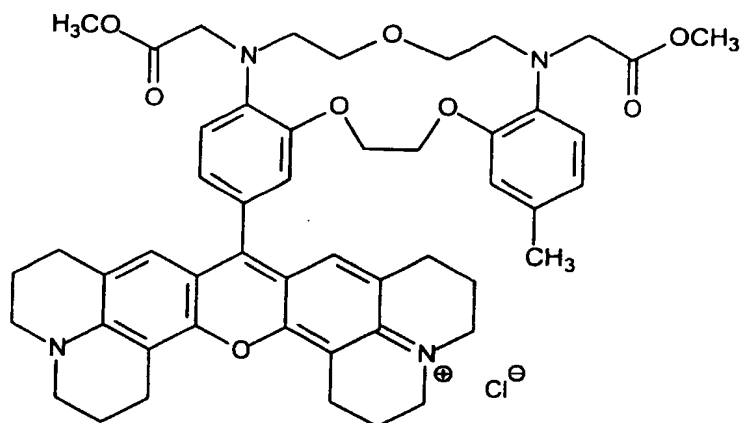
Compound 140

Example 25. Preparation of 1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[X-Rhd]-DDTCPD (143).

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Compound 143 was prepared analogously to Compound 138, using 8-hydroxyjulolidine rather than *m*-dimethylaminophenol.

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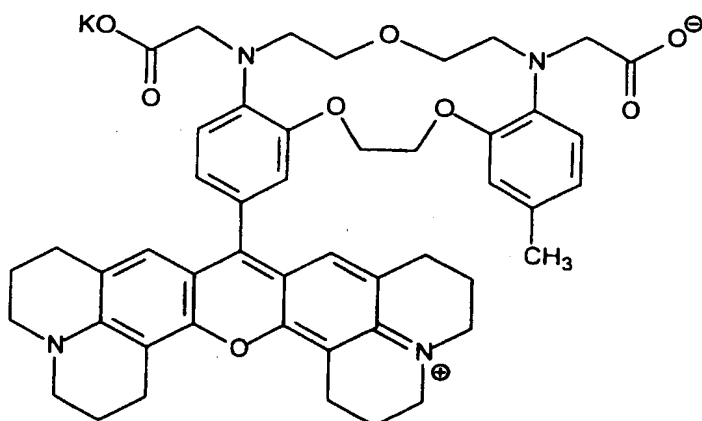


Compound 143

Example 26. Preparation of 1,18-bis(carboxymethyl)-14-methyl-5-[X-Rhd]-DDTCPD potassium salt (144).

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Compound 144 was prepared from Compound 138 using the procedure for preparing Compound 139 from Compound 138.



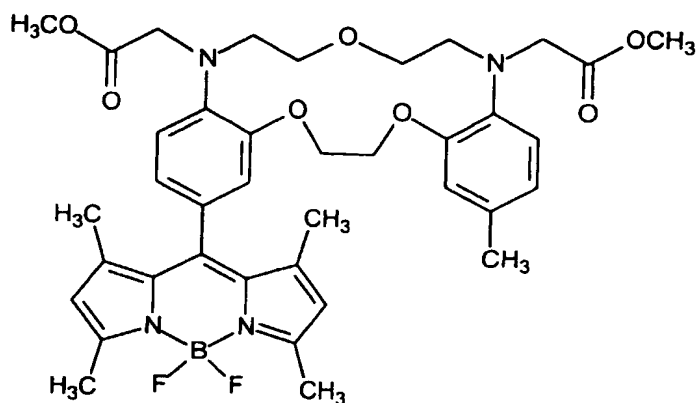
Compound 144

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Example 27. Preparation of 1,18-bis(carboxymethyl)-14-methyl-5-[BODIPY]-DDTCPD (145).

To a solution of aldehyde **109** (250 mg, 0.5 mmol) in CH₂Cl₂ (20 mL) was added 2,4-dimethylpyrrole (0.125 mL, 1.2 mmol). The solution was stirred for 5 min, then TFA (0.046 mL, 0.6 mmol) is introduced. After 16 h, the mixture was diluted with CHCl₃ (150 mL), washed with 2% tetrabutylammonium hydroxide (150 mL) then H₂O then evaporated. The residue was dissolved in toluene (20 mL) and stirred for 3 h with chloranil (148 mg, 0.6 mmol). DIPEA (0.87 mL, 5 mmol) was introduced, followed by BF₃ etherate (0.52 mL, 4 mmol). The mixture was evaporated and the residue was purified by chromatography on SiO₂ using a gradient of 0-2% MeOH in CHCl₃ to give compound **145**, 102 mg (28%) as a brown solid.

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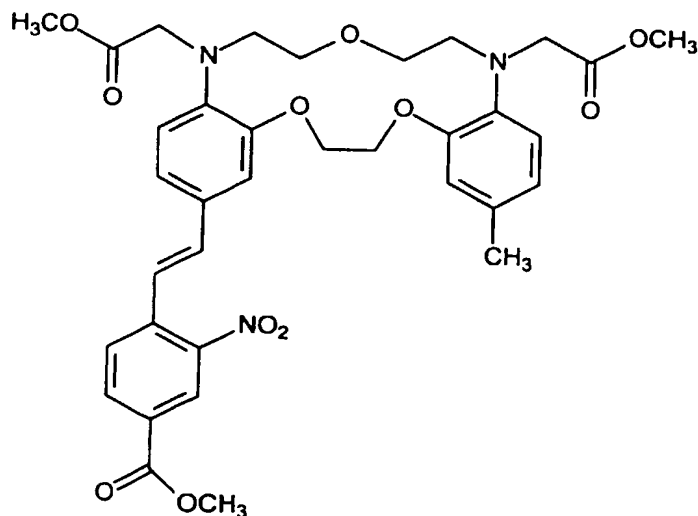
Compound 145

Example 28. Preparation of trans 1-[1',18'-bis-(methoxycarbonylmethyl)-14'-methyl-DDTCPD-5-yl]-2-[4''-methoxycarbonyl-2''-nitrophenyl] ethylene (147).

5

A mixture of aldehyde **109** (1.00 g, 2.0 mmol), the Wittig base (4-methoxycarbonyl-2-nitrobenzyl)triphenylphosphonium bromide (1.34 g, 2.5 mmol), and K_2CO_3 (1.38 g, 10 mmol) in DMF (20 mL) was stirred for 16 h at 95 °C. More of the Wittig base (0.500 g, 0.93 mmol) was added and the mixture was stirred for an additional 6 h, then cooled to room temperature and poured into H_2O . The solution was acidified with 1 M HCl to pH 3, extracted with $CHCl_3$, dried over $MgSO_4$, and evaporated. The crude product was purified by chromatography on SiO_2 using $CHCl_3$ then 2% MeOH in $CHCl_3$ to give compound **147**, 0.915 g (68%) as an orange solid.

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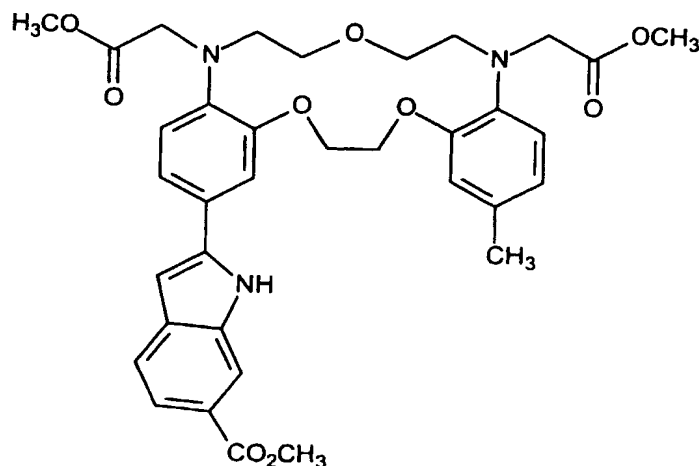
15

Compound 147

Example 29. Preparation of 1,18-bis(methoxycarbonylmethyl)-5-(6'-methoxycarbonylindolyl)-2'-14-methyl-DDTCPD (148).

A mixture of the ethylene derivative 147 (70 mg, 0.1 mmol) and triethylphosphite (3 mL) was heated at 120 °C for 6 h, then evaporated and subsequently co-evaporated with DMF (3 x 10 mL). The residue was purified by preparative TLC on two SiO₂ plates using 7% MeOH in CHCl₃ as eluant to give compound 148, 52 mg (80%) as a slightly yellowish solid.

5



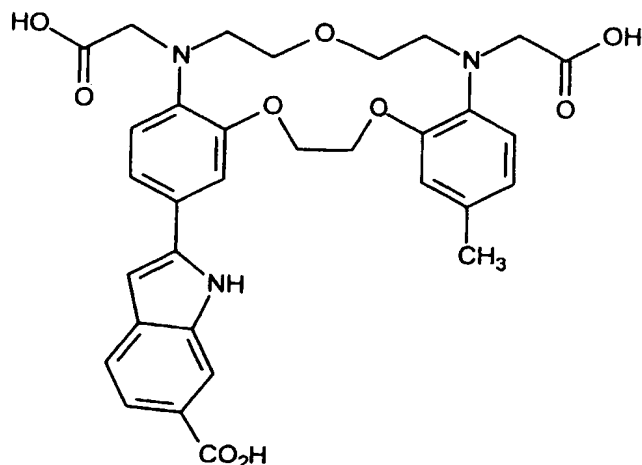
Compound 148

Example 30. Preparation of 1,18-bis(carboxymethyl)-5-(6'-carboxy indolyl-2')-14-methyl-DDTCPD (149).

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A mixture of Compound 148 (300 mg, 0.46 mmol), and 1 M KOH (6 mL, 6 mmol) in MeOH (25 mL) was stirred for 16 h. More 1 M KOH (5 mL, 5 mmol) was added and the mixture was stirred for an additional 6 h, then evaporated to half its original volume, and acidified with 1 M HCl to pH 3. The suspension was extracted with CHCl₃ then with n-BuOH. The combined organic extract was filtered and was evaporated. Ether (20 mL) was added and the precipitated solid was filtered and washed with ether to give compound 149, 157 mg (57%) as an off-white solid.

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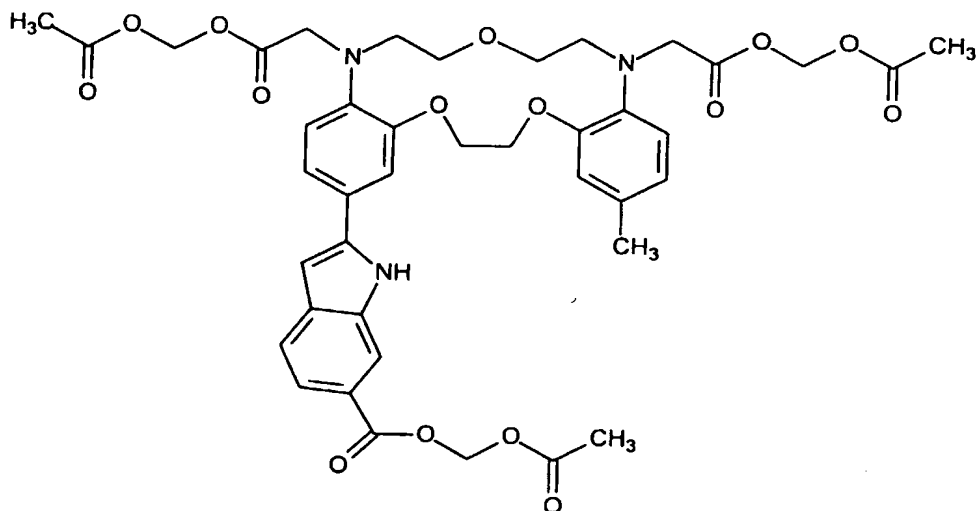


Compound 149

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Example 31. Preparation of 1,18-bis(acetoxymethoxycarbonylmethyl)-5-(6'-acetoxymethoxycarbonylmethyl)-14-methyl-DDTCPD (150).

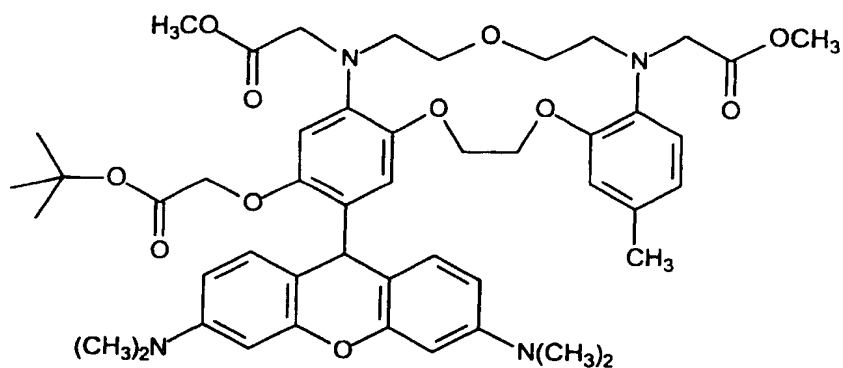
5 A mixture of Compound 149 (72 mg, 0.12 mmol), bromomethyl acetate (0.14 mL, 1.0 mmol), and DIPEA (0.35 mL, 2 mmol) in DMF (5 mL) was stirred for 16 h, poured into 1% AcOH (200 mL), extracted with CHCl₃, washed with H₂O then evaporated. The residue was purified by preparative TLC on two SiO₂ plates using 5% MeOH in CHCl₃ to give Compound 150, 3 mg (3%) as a brown solid.



Compound 150

Example 32. Preparation of 4-(*t*-butoxycarbonylmethoxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[2H-TMR]-DDTCPD (151).

15 A mixture of aldehyde 119 (630 mg, 1.0 mmol), 3-dimethylaminophenol (330 mg, 2.4 mmol), and *p*-toluenesulphonic acid (20 mg, catalyst) in propionic acid (5 mL) was stirred for 16 h at 60 °C, then cooled to room temperature and poured into 3 M NaOAc (150 mL). The precipitated solid was filtered, washed with H₂O and dried to give Compound 151, 360 mg (97%) as a rose solid. Compound 151 is unstable to oxidation and was used in next step
20 without additional purification.



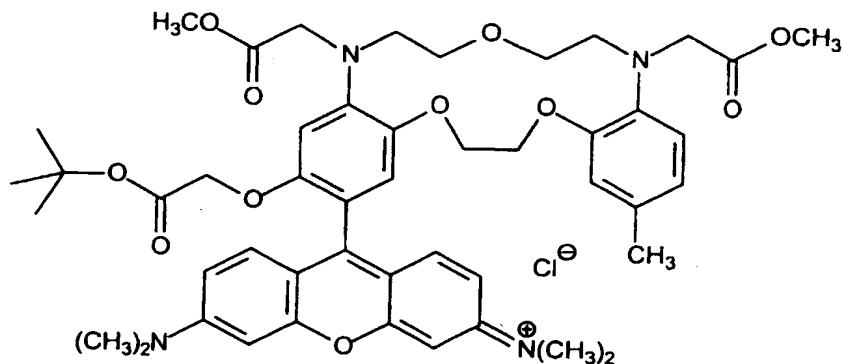
Compound 151

Example 33. Preparation of 4-(*t*-butoxycarbonylmethoxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (152).

5

A mixture of Compound 151 (2.50 g, 2.9 mmol) and chloranil (1.42 g, 5.8 mmol) in MeOH (100 mL) and CHCl₃ (100 mL) was stirred for 4 h, filtered from excess oxidizer, and evaporated. The residue was purified by chromatography on SiO₂ using a gradient of 6-10% MeOH and 1% AcOH in CHCl₃ to give compound 152, 1.13 g (45%) as a crimson solid.

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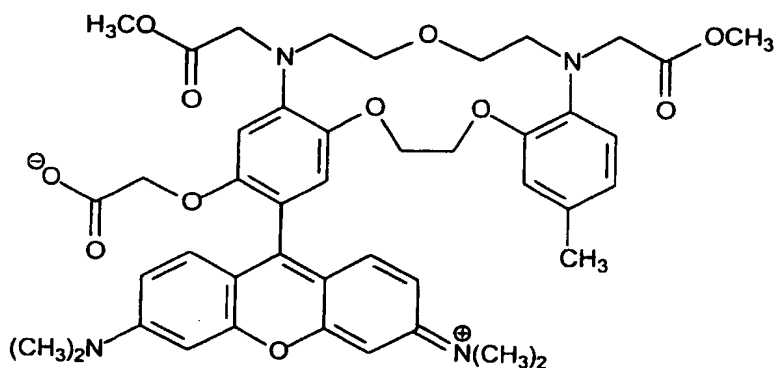
Compound 152

Example 34. Preparation of 4-carboxymethoxy-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (153).

15

A mixture of Compound 152 (500 mg, 0.58 mmol) in CH₂Cl₂ (20 mL) and TFA (20 mL) was stirred for 4 h, then evaporated and co-evaporated with CHCl₃. Ether (25 mL) was added to the residue and the precipitated product was filtered and washed with ether to give Compound 153, 447 mg (95%) as a violet-red solid.

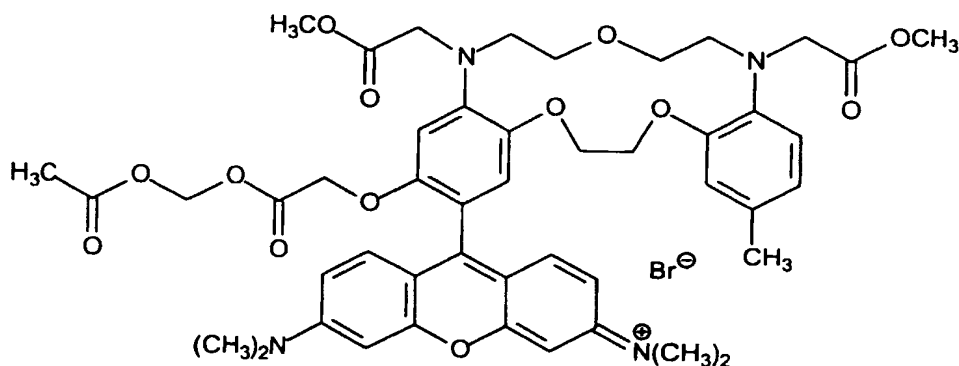
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Compound 153

Example 35. Preparation of 4-acetoxymethoxycarbonylmethoxy-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (154).

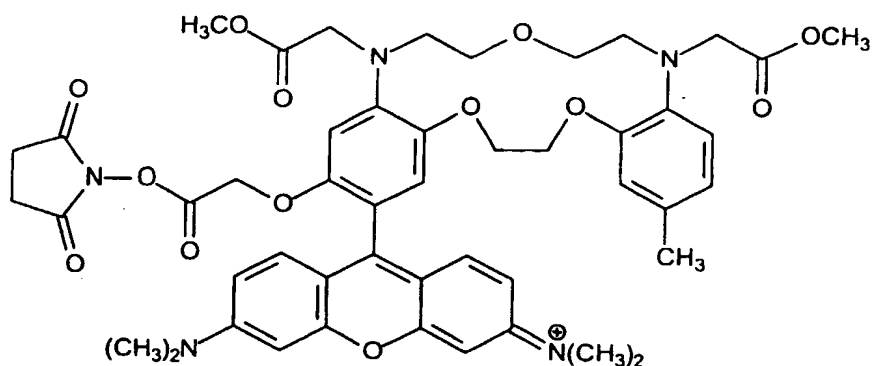
5 A mixture of Compound 153 (20 mg, 0.1 mmol), bromomethyl acetate (0.025 mL, 0.25 mmol), and DIPEA (0.08 mL, 0.5 mmol) in DMF (1 mL) was stirred for 3 h. More bromomethyl acetate (0.025 mL, 0.25 mmol) and DIPEA (0.08 mL, 0.5 mmol) were added and the mixture was stirred for an additional 3 h, diluted with CHCl₃ (100 mL), washed with 1% AcOH then
 10 H₂O then evaporated. The residue was purified by preparative TLC on two SiO₂ plates using 10% MeOH and 0.5% AcOH in CHCl₃ to give Compound 154, 12 mg (54%) as a dark red solid.



Compound 154

Example 36. Preparation of 4-carboxymethoxy-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD, succinimidyl ester (155).

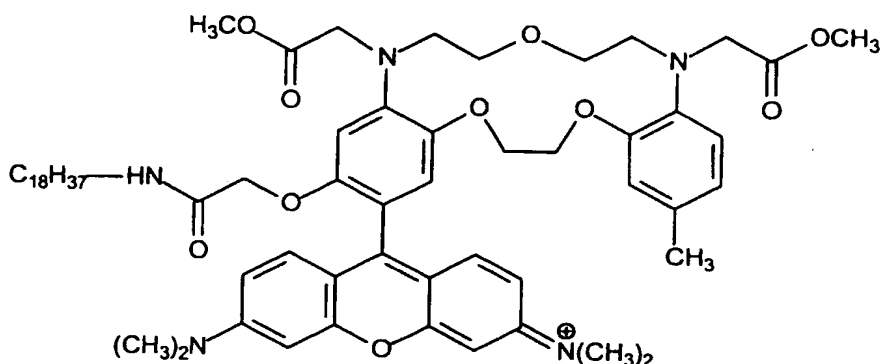
To a stirred solution of Compound 153 (162 mg, 0.2 mmol) and pyridine (0.08 mL, 1 mmol) in DMF (5 mL), was added dry N-trifluoroacetoxysuccinimide (90 mg, 0.4 mmol). After 7 h more
 20 N-trifluoroacetoxysuccinimide (90 mg, 0.4 mmol) and pyridine (0.08 mL, 1 mmol) were added, and the mixture was stirred for 16 h more. Analytical TLC confirmed the formation of the single product, while starting material 153 was consumed. Compound 155 was very reactive and unstable towards isolation attempts. It was used in further transformations upon preparation in DMF solution without isolation and purification.



Compound 155

Example 37. Preparation of 4-(N-hexadecylaminocarbonylmethoxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (156).

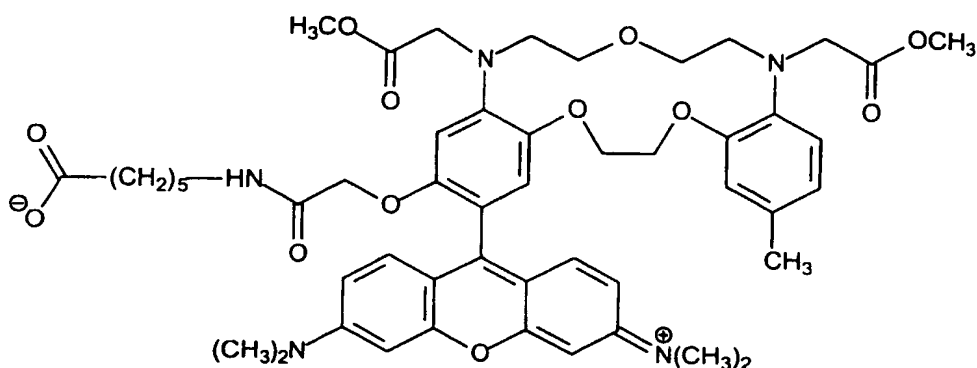
5 To a stirred solution of succinimidyl ester 155 prepared as in Example 36 from compound 153 (81 mg, 0.1 mmol) in DMF (2 mL) was added dry hexadecylamine (135 mg, 0.5 mmol). The mixture was stirred for 16 h, diluted with CHCl₃ (150 mL), washed with 1% AcOH the H₂O, dried over MgSO₄, and evaporated. The residue was purified by preparative TLC on two SiO₂ plates using CHCl₃ as eluant to give Compound 156, 57 mg (54%) as a red oil.



Compound 156

15 **Example 38. Preparation of 4-(N-5'-carboxypentylaminocarbonylmethoxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (157).**

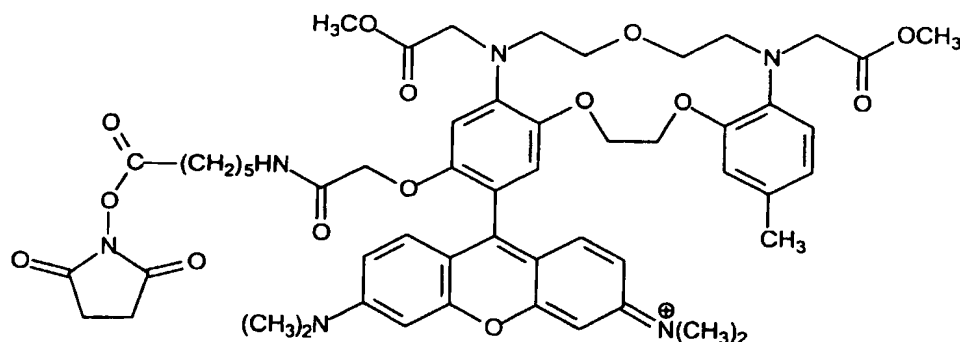
20 To a stirred solution of succinimidyl ester 155 prepared as in Example 36 from Compound 153 (81 mg, 0.1 mmol) in DMF (2 mL) was quickly introduced a solution of 5-aminopentanoic acid (40 mg, 0.3 mmol) and 1 M methanolic tetrabutylammonium hydroxide (1 mL, 1 mmol) in H₂O (5 mL). The mixture was stirred for 4 h, then diluted with H₂O (100 mL), acidified with AcOH to pH 4, extracted with CHCl₃, dried over MgSO₄ and evaporated. The residue was purified by preparative TLC on two SiO₂ plates using 10% MeOH and 2.5% AcOH in CHCl₃ as the eluant to give Compound 157, 56 mg (61%) as a red solid.



Compound 157

Example 39. Preparation of 4-(N-5'-Carboxypentylaminocarbonylmethoxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD, succinimidyl ester (158).

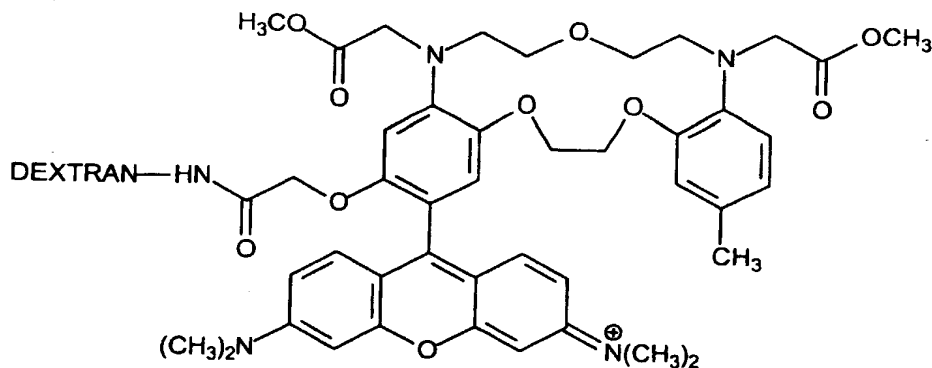
5 To a stirred solution of Compound 157 (37 mg, 0.04 mmol) and pyridine (0.08 mL, 1 mmol) in DMF (1 mL), was added dry N-trifluoroacetoxy succinimide (36 mg, 0.16 mmol). After 4 h, more N-trifluoroacetoxy succinimide (36 mg, 0.4 mmol) and pyridine (0.08 mL, 1 mmol) are added, and the mixture was stirred for an additional 16 h. The mixture was diluted with
10 CHCl₃ (100 mL), washed with 1% AcOH then H₂O then evaporated. Hexanes (5 mL) were added to the residue. The precipitated solid was filtered, washed with hexanes and dried to give Compound 158, 15 mg (37%) as a dark red solid.



Compound 158

Example 40. Preparation of 4-carboxymethoxy-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD, dextran conjugate (160).

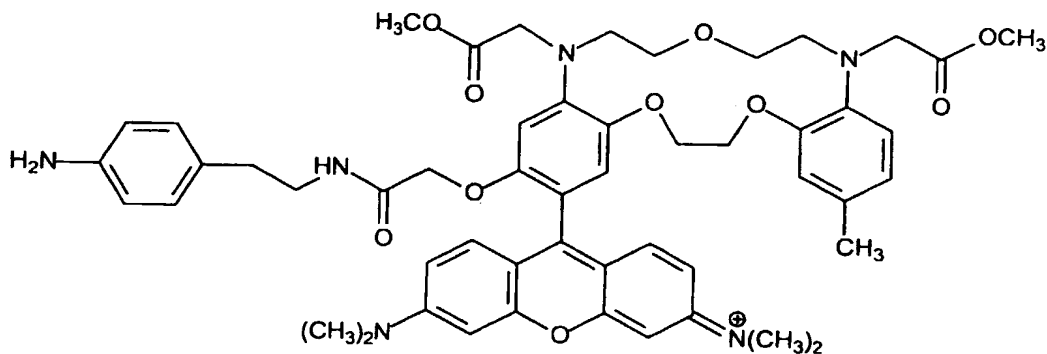
15 A solution of succinimidyl ester 155 (81 mg, 0.1 mmol) in DMF (2 mL) was added to 5 mL of aqueous aminodextran (100 mg, 0.037 eq.) and 1 M methanolic tetrabutylammonium hydroxide (1 mL, 1 mmol). The mixture was stirred for 16 h, poured into MeOH (400 mL), and the precipitated conjugate was filtered off and washed with MeOH. The crude product was dissolved in H₂O (3 mL), filtered through a membrane filter and loaded onto a SEPHADEX G-
20 15 resin column pre-equilibrated with H₂O. The colored fraction was eluted in a void volume and lyophilized to give labeled dextran 160, (41 mg) as a red solid.
25



Compound 160

5 Example 41. Preparation of 4-(N-[(4"-aminophenyl)ethyl-2']carbonylmethyloxy)-1,18-
bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (161).

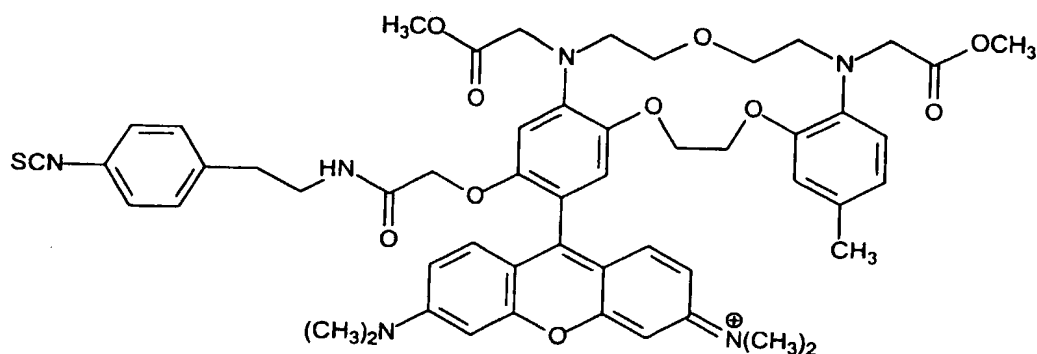
10 To a stirred solution of succinimidyl ester 155 (81 mg, 0.1 mmol) in DMF (2 mL) was added Et₃N (0.14 mL, 1 mmol) followed by 2-(4-aminophenyl)ethylamine (0.05 mL, 0.5 mmol). The mixture was stirred for 2 h, diluted with CHCl₃ (150 mL), washed with 1% AcOH then H₂O then evaporated. Ether (5 mL) was added to the residue, and the solid was filtered off, washed with ether and dried to give Compound 161 as a dark red solid, pure on TLC and HPLC.



Compound 161

15 Example 42. Preparation of 4-(N-[(4"-isothiocyanatophenyl)ethyl-2']carbonylmethyloxy)-
1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (162).

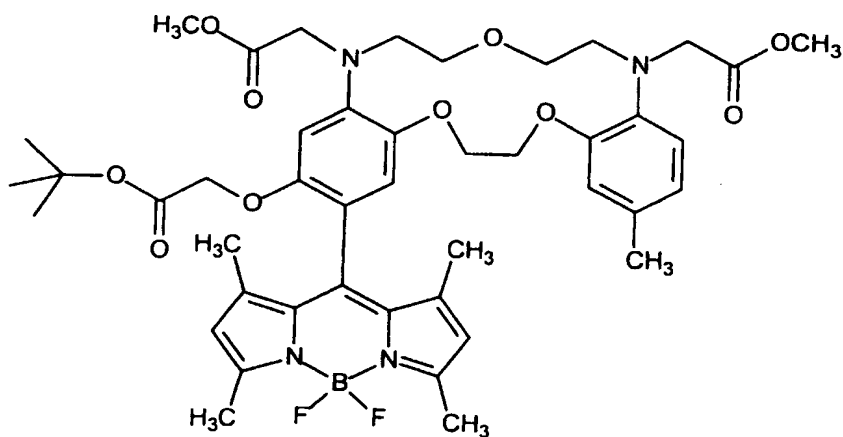
20 To a solution of Compound 161 (9 mg, 0.01 mmol) in AcOH (2 mL) was added a 1 M CSCl₂ solution in CHCl₃ (0.1 mL, 0.1 mmol). The mixture was stirred for 2 h then evaporated. Ether (5 mL) was added to the residue, and the precipitated solid was filtered off, washed with ether and dried to give Compound 162, 9 mg (95%) as a dark red solid. Compound 162 reacts quickly with n-BuNH₂ in pre-column derivatization for HPLC analysis.



Compound 162

5 Example 43. Preparation of 4-(*t*-butoxycarbonylmethoxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen-8-yl]-DDTCPD (163).

To a stirred solution of aldehyde 119 (630 mg, 1.0 mmol) in CH₂Cl₂ (40 mL), 2,4-dimethylpyrrole (0.25 mL, 2.4 mmol) was added. The solution was stirred for 5 min, then TFA (0.1 mL, 1.2 mmol) was introduced. The mixture was stirred for 16 h, and diluted with CHCl₃ (300 mL). The chloroform solution was washed with 2% tetrabutylammonium hydroxide then H₂O, evaporated, and subsequently co-evaporated with toluene. The residue was dissolved in toluene (40 mL), stirred for 2 h with chloranil (296 mg, 1.2 mmol), then DIPEA (1.74 mL, 10 mmol) was introduced, followed by BF₃ etherate (1.04 mL, 8 mmol). The mixture was
15 evaporated and the residue was purified by chromatography on SiO₂ using a gradient of 0-1% MeOH in CHCl₃ to give Compound 163, 280 mg (35%) as a brown solid.

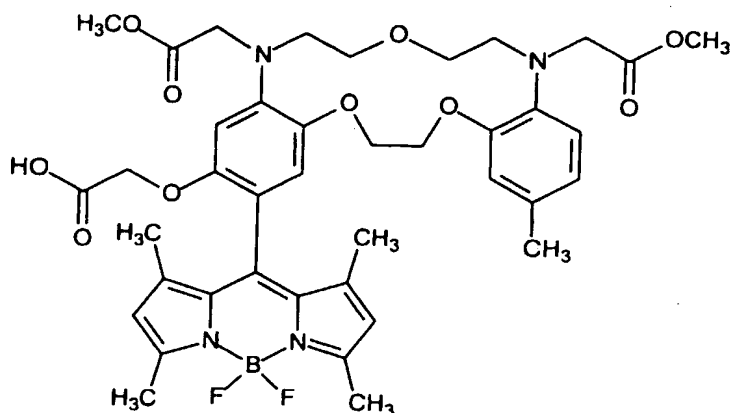


Compound 163

20 Example 44. Preparation of 4-(*t*-butoxycarbonylmethoxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen-8-yl]-DDTCPD (164).

A mixture of Compound 163 (120 mg, 0.15 mmol) in CHCl_3 (6 mL) and TFA (0.4 mL) was stirred for 16 h, then evaporated and co-evaporated with CHCl_3 . The residue was purified by preparative TLC on two SiO_2 plates using 10% MeOH in CHCl_3 as eluant to give Compound 164, 82 mg (77%) as a brown solid.

5



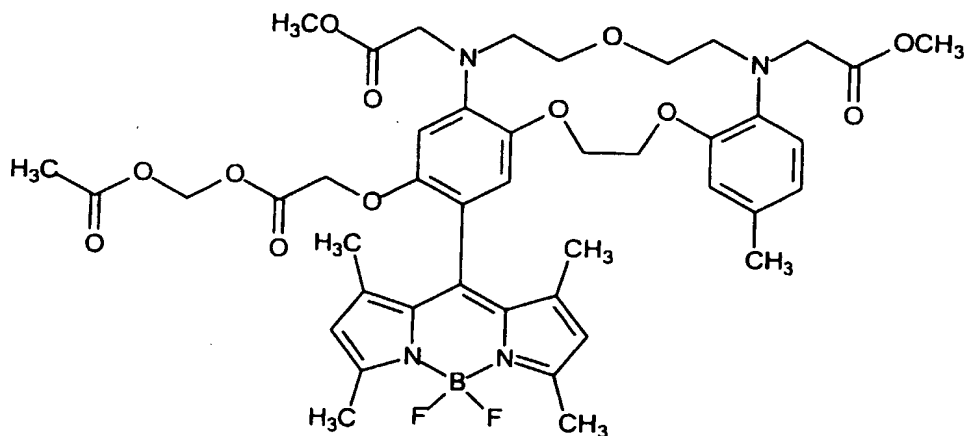
Compound 164

Example 45. Preparation of 4-acetoxymethoxycarbonylmethoxy-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diazas-indacen-8-yl]-DDTCPD (165).

10

A mixture of Compound 164 (80 mg, 0.11 mmol), bromomethyl acetate (0.05 mL, 0.5 mmol), and DIPEA (0.17 mL, 1.0 mmol) in DMF (2 mL) was stirred for 2 h, poured into H_2O (150 mL), and extracted with CHCl_3 . The extract was dried over MgSO_4 then evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 3% MeOH and 0.5% AcOH in CHCl_3 to give Compound 165, 46 mg (52%) as a brown solid.

15

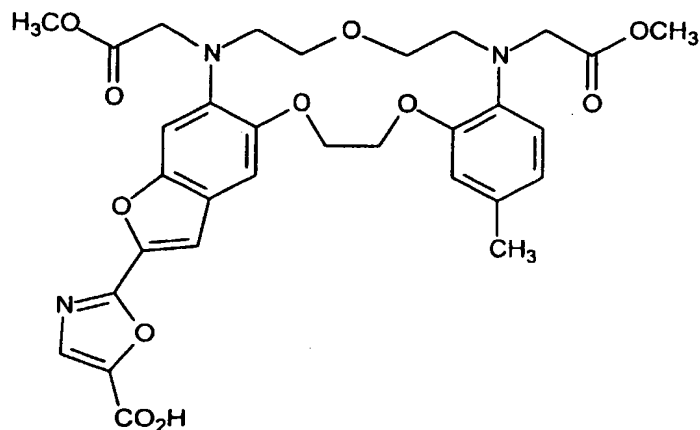


Compound 165

Example 46. Preparation of Compound 167.

20

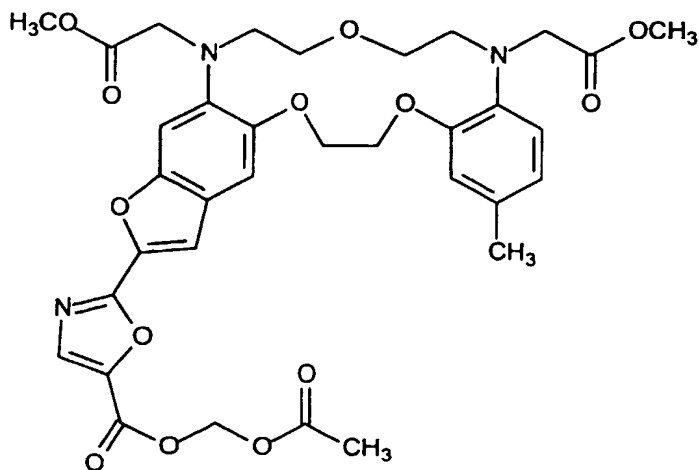
A mixture of aldehyde 118 (800 mg, 1.55 mmol), 5-carboxy-2-chloromethylthiazole (300 mg, 1.86 mmol), K_2CO_3 (1.07 g, 7.75 mmol), and NaI (75 mg, 0.5 mmol; catalyst) in DMF (20 mL) was stirred at 135 °C for 4 h, then poured into H_2O . The mixture was acidified with 1 M HCl to pH 3, extracted with $CHCl_3$, dried over $MgSO_4$ then evaporated. The residue was purified by chromatography on SiO_2 using a gradient of 5–20% MeOH and 1–2% AcOH in $CHCl_3$ as eluant to give Compound 167, 255 mg (26%) as a yellowish solid.



Compound 167

Example 47. Preparation of Compound 168.

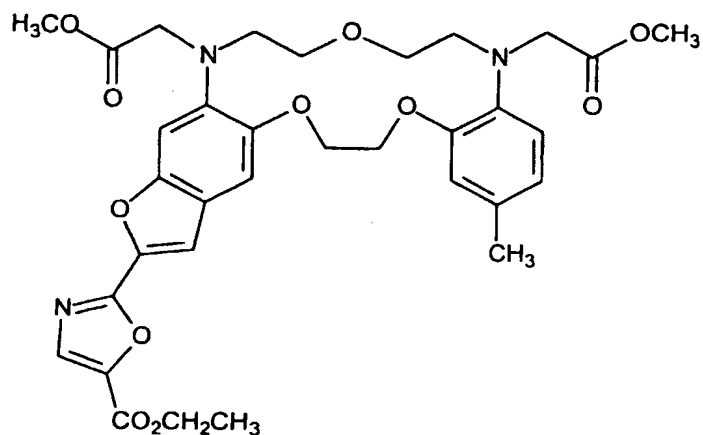
A mixture of Compound 167 (62 mg, 0.1 mmol), bromomethyl acetate (0.05 mL, 0.5 mmol), and DIPEA (0.17 mL, 1.0 mmol) in DMF (1 mL) was stirred for 2 h, poured into 1% AcOH (100 mL), and extracted with $CHCl_3$. Then 3 M NaOAc (100 mL) was added to the aqueous phase and the mixture was extracted with more $CHCl_3$. The combined extracts were dried over $MgSO_4$ then evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 5% MeOH in $CHCl_3$ to give Compound 168, 29 mg (42%) as an orange solid.



Compound 168

Example 48. Preparation of Compound 170.

5 A mixture of aldehyde 118 (1.40 g, 2.71 mmol), 2-chloromethyl-5-ethoxycarbonyloxazol (0.564 g, 1.86 mmol), K_2CO_3 (1.87 g, 13.60 mmol), and NaI (0.150 g, 1.00 mmol; catalyst) in DMF (30 mL) was stirred at 135 °C for 4 h, then poured into H_2O . The mixture was acidified with 1 M HCl to pH 3, extracted with $CHCl_3$ and evaporated. The residue was purified by chromatography on SiO_2 using 1% MeOH in $CHCl_3$ as eluant to give Compound 170, 1.260 g (71%) as a yellowish solid.

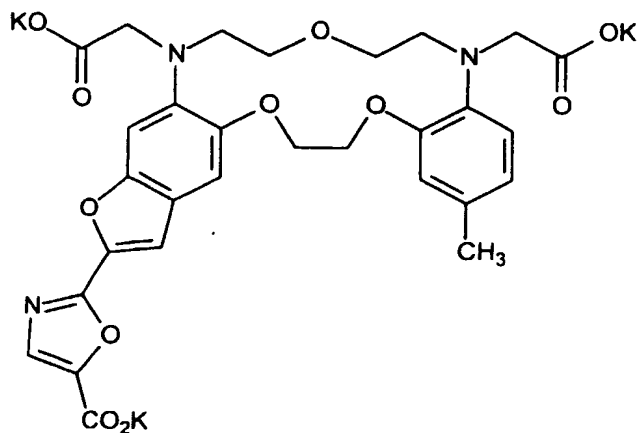


Compound 170

Example 49. Preparation of Compound 171.

15 A mixture of Compound 170 (376 mg, 0.5 mmol) and 1 M KOH (3 mL, 3.0 mmol) in MeOH (5 mL) was stirred for 5 h, then evaporated to 1/5 volume, and acidified with 1 M HCl to pH 2.8. The precipitated solid was filtered, washed with H_2O and dried on a filter. This crude product was suspended in H_2O (2 mL), then made basic with 0.1 M KOH to pH 9.5. The solution was loaded onto a SEPHADEX LH-20 resin column and chromatographed using H_2O as eluant to give Compound 171, 256 mg (72%) as a yellow-greenish solid.

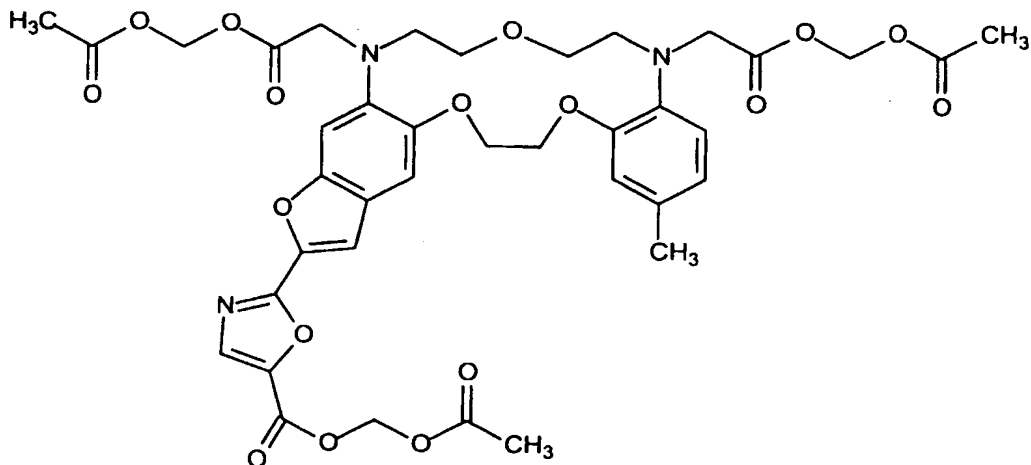
20



Compound 171

Example 50. Preparation of Compound 172.

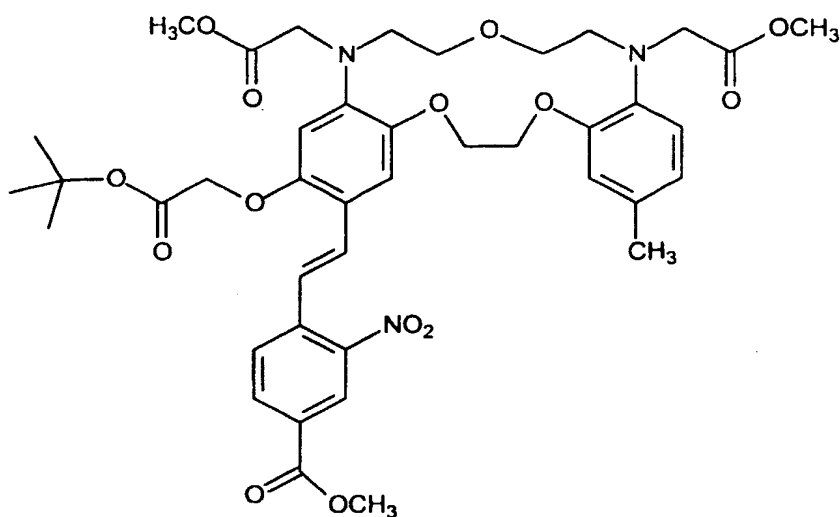
5 A mixture of Compound 171 (71 mg, 0.1 mmol), bromomethyl acetate (0.1 mL, 1.0 mmol), and DIPEA (0.35 mL, 2.0 mmol) in DMF (2 mL) was stirred for 4 h, diluted with CHCl₃, then washed with 1% AcOH then H₂O and evaporated. The residue was purified by preparative TLC on two SiO₂ plates using 5% MeOH and 0.5% AcOH in CHCl₃ to give compound 172, 63 mg (77%) as an orange solid.



Compound 172

Example 51. Preparation of trans 1-[4'-*t*-butoxycarbonylmethoxy-1',18'-bis-(methoxycarbonylmethyl)-14'-methyl-DDTCPD-5-yl]-2-[4"-methoxycarbonyl-2"-nitrophenyl]ethylene (173).

20 A mixture of aldehyde 119 (315 mg, 0.5 mmol), the Wittig base (4-methoxycarbonyl-2-nitrobenzyl)triphenylphosphonium bromide (375 mg, 0.7 mmol), and K₂CO₃ (345 mg, 2.5 mmol) in DMF (3 mL) was stirred for 6 h at 95 °C then cooled to room temperature and poured into H₂O. The solution was acidified to pH 5 with 1 M HCl and extracted with CHCl₃. The extract was dried over MgSO₄ and evaporated. The crude product was purified by chromatography on SiO₂ using CHCl₃, then 0.5% MeOH in CHCl₃ to give Compound 173, 339 mg (84%) as an orange solid.



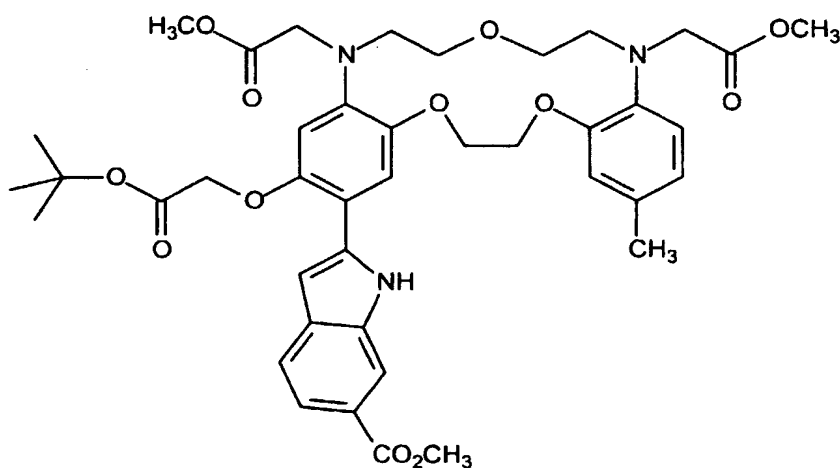
Compound 173

Example 52. Preparation of 4-*t*-butoxycarbonylmethoxy 1,18-bis-(methoxycarbonylmethyl)-5-(6'-methoxycarbonylindolyl-2')-14-methyl-DDTCPD (174).

5

A mixture of the ethylene derivative 173 (338 mg, 0.42 mmol) and triethylphosphite (8 mL) was heated at 130 °C for 7 h, then evaporated and subsequently co-evaporated with DMF. The residue was first purified by chromatography on SiO₂ using CHCl₃ followed by 1% MeOH in CHCl₃, then by preparative TLC on two SiO₂ plates using 50% EtOAc in hexanes as eluant to give Compound 174, 112 mg (34%) as a slightly yellowish solid.

10



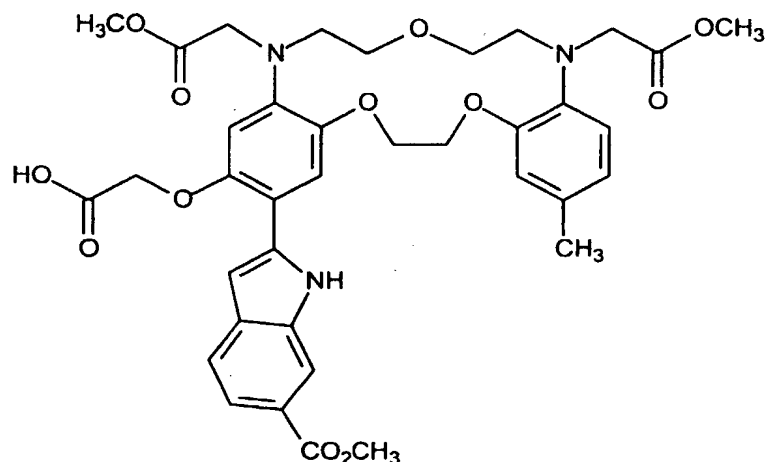
Compound 174

Example 53. Preparation of 4-carboxymethoxy 1,18-bis-(methoxycarbonylmethyl)-5-(6'-methoxycarbonylindolyl-2')-14-methyl-DDTCPD (175).

15

A mixture of Compound 174 (104 mg, 0.13 mmol) in CH₂Cl₂ (2 mL) and TFA (2 mL) was stirred for 3 h, then evaporated and co-evaporated with CHCl₃. The residue was purified by

preparative TLC on two SiO₂ plates using 7% MeOH and 2% AcOH in CHCl₃ to give Compound 175, 55 mg (57%) as a yellowish solid.

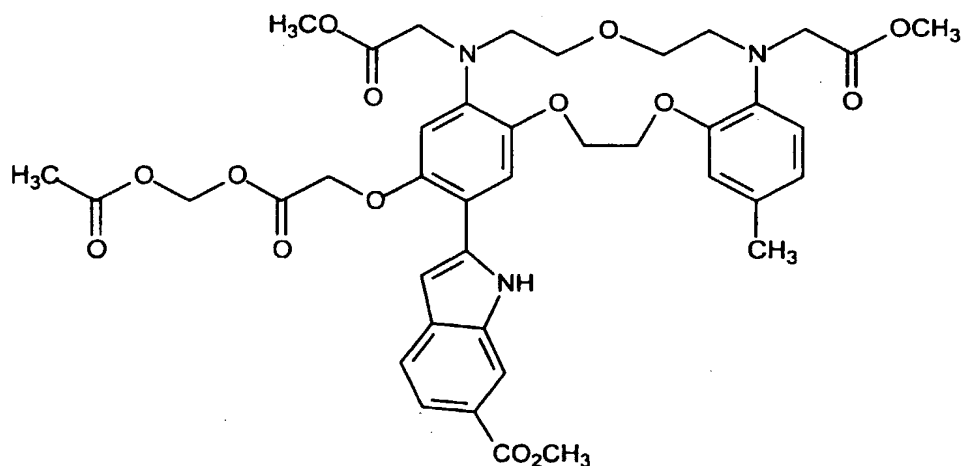


Compound 175

5

Example 54. Preparation of 4-acetoxymethylcarbonylmethoxy 1,18-bis-(methoxycarbonylmethyl)-5-(6'- methoxycarbonylindolyl)-2'-14-methyl-DDTCPD (176).

A mixture of Compound 175 (22 mg, 0.03 mmol), bromomethyl acetate (0.1 mL, 1.0 mmol), and DIPEA (0.26 mL, 1.5 mmol) in DMF (2 mL) was stirred for 16 h, then diluted with CHCl₃ (100 mL), washed with 1% AcOH then H₂O then evaporated. The residue was purified by preparative TLC on two SiO₂ plates using 5% MeOH in CHCl₃ to give compound 176, 16 mg (67%) as an orange solid.



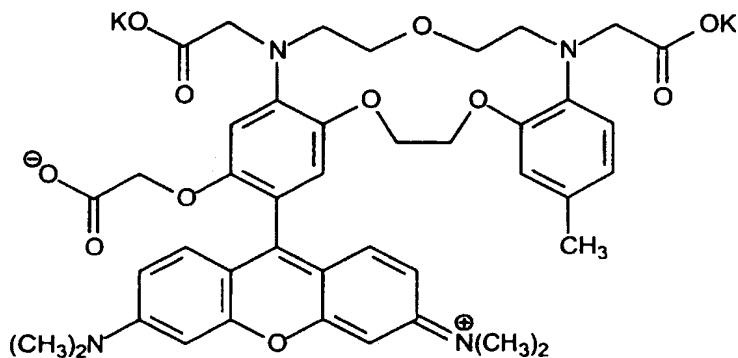
Compound 176

15

Example 55. Preparation of 4-carboxymethoxy-1,18-bis(carboxymethyl)-14-methyl-5-[TMR]-DDTCPD (177).

A mixture of Compound 152 (87 mg, 0.1 mmol) and 1 M KOH (2 mL, 2 mmol) in MeOH (5 mL) was stirred for 16 h, and evaporated. The residue was purified on SEPHADEX LH-20 resin using H₂O as the eluant. The fractions containing product were collected and lyophilized to give Compound 177, 12 mg (14%) as a crimson solid.

5



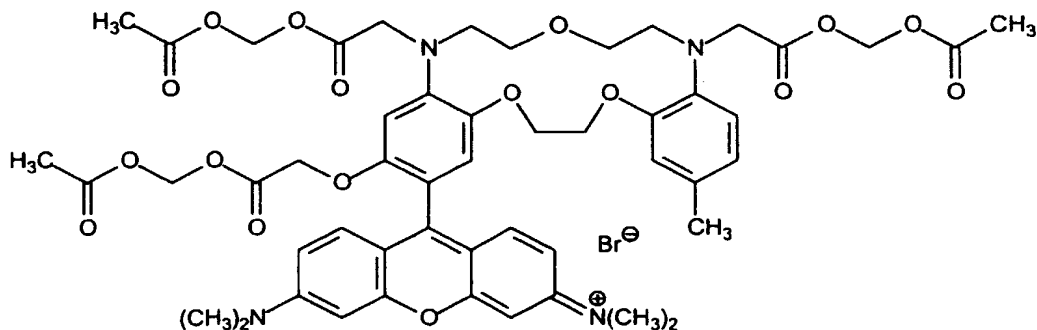
Compound 177

Example 56. Preparation of 4-acetoxymethoxycarbonylmethyloxy-1,18-bis(acetoxymethoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (178).

10

A mixture of Compound 177 (8 mg, 0.01 mmol), bromomethyl acetate (0.025 mL, 0.25 mmol), and DIPEA (0.08 mL, 0.5 mmol) in DMF (1 mL) was stirred for 16 h, and diluted with CHCl₃ (100 mL). The solution was washed with 1% AcOH then H₂O then evaporated. Ether (5 mL) was added to the residue. The precipitated solid was filtered off and washed with ether to give Compound 178, 5 mg (50%) as a dark red solid. Compound 178 was pure on TLC and HPLC.

15



Compound 178

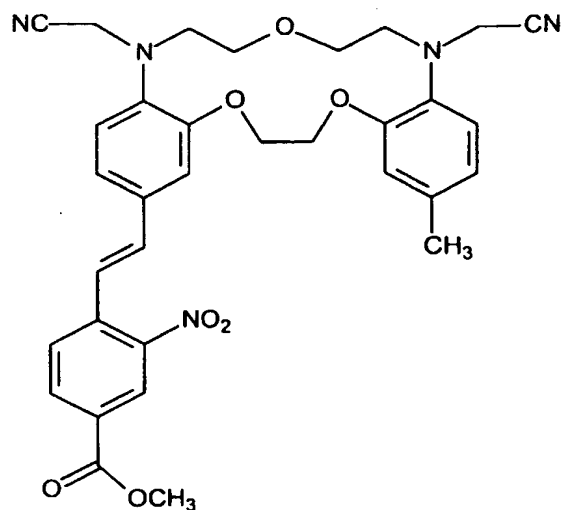
Example 57. Preparation of trans 1-[1',18'-bis-(cyanomethyl)-14'-methyl-DDTCPD-5-yl]-2-[4'-methoxycarbonyl-2''-nitrophenyl] ethylene (179).

20

A mixture of aldehyde 121 (217 mg, 0.5 mmol), the Wittig base (4-methoxycarbonyl-2-nitrobenzyl)triphenylphosphonium bromide (536 mg, 0.7 mmol), and K₂CO₃ (345 mg, 2.5 mmol) in DMF (3 mL) was stirred for 16 h at 95 °C, then cooled to room temperature and poured into H₂O. The solution was acidified with 1 M HCl to pH 3, extracted with CHCl₃,

25

dried over MgSO_4 and evaporated. The crude product was purified by chromatography on SiO_2 using CHCl_3 , as eluant to give Compound 179, 171 mg (56%) as an orange solid.

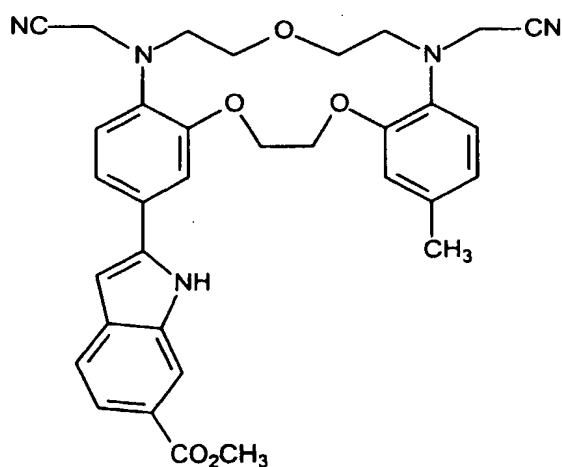


Compound 179

5

Example 58. Preparation of 1,18-bis(cyanomethyl)-5-(6'-methoxycarbonylindolyl-2')-14-methyl-DDTCPD (180).

A mixture of the ethylene derivative 179 (170 mg, 0.28 mmol) and triethylphosphite (4 mL) was heated at 120 °C for 16 h, then evaporated and subsequently co-evaporated with DMF. The residue was purified by preparative TLC on two SiO_2 plates using 3% MeOH in CHCl_3 as eluant, then on two SiO_2 plates using 40% EtOAc in hexanes as eluant to give Compound 180, 26 mg (16%) as a yellowish solid.

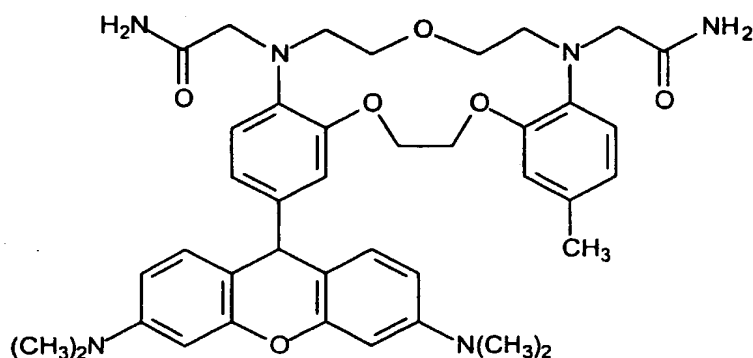


Compound 180

15

Example 59. Preparation of 1,18-bis(carbamoylmethyl)-14-methyl-5-[dihydrotetramethylrhodamine]-DDTCPD (181).

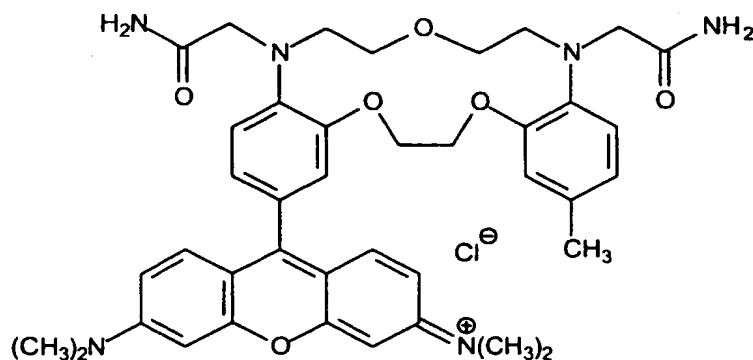
A mixture of aldehyde 123 (100 mg, 0.21 mmol), 3-dimethylaminophenol (55 mg, 0.40 mmol), and p-toluenesulphonic acid (5 mg, catalyst) in propionic acid (2 mL) was stirred overnight at 60 °C, then cooled to room temperature and poured into 3 M NaOAc (40 mL). Precipitated solid was filtered, washed with H₂O and dried to give Compound 181, 140 mg (93%) as a rose-colored solid. Compound 181 was unstable to oxidation and was used in next step without additional purification.



Compound 181

Example 60. Preparation of 1,18-bis(carbamoylmethyl)-14-methyl-5-[tetramethylrhodamine]-DDTCPD (182).

A mixture of Compound 181 (138 mg, 0.20 mmol) and chloranil (84 mg, 0.34 mmol) in MeOH (5 mL) and CHCl₃ (5 mL) was stirred for 4 h, and evaporated. The residue was purified by chromatography on SiO₂ using a gradient of 7-9% MeOH and 0.5-1% AcOH in CHCl₃ as eluant to give Compound 182, 118 mg (86%) as a dark red solid.

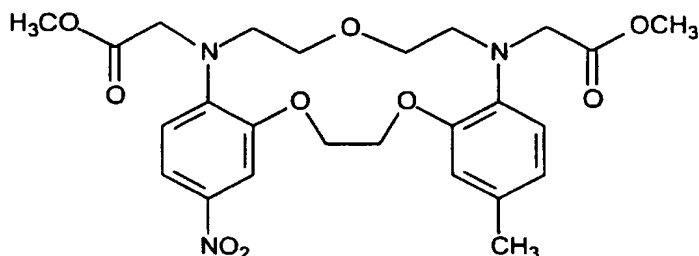


Compound 182

Example 61. Preparation of Compound 200.

To a 0.5 M solution of arene 108 in sulfuric acid is added one equivalent of potassium nitrate. The resulting solution is stirred until TLC analysis of reaction aliquots, treated with water

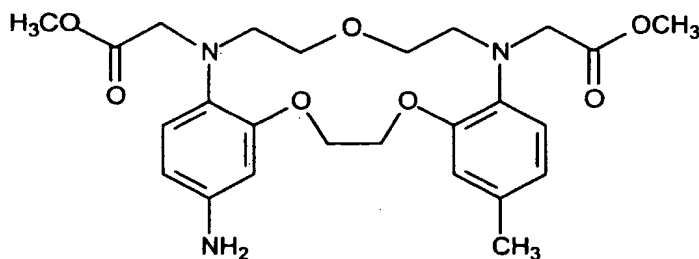
and ether, shows full conversion to nitroarene **200**. The reaction mixture is poured into excess aqueous sodium acetate, followed by extraction with ether. The extract is washed with aqueous sodium bicarbonate and brine, dried over magnesium sulfate, then concentrated to a reddish-brown oil, which is purified by trituration with ether-hexanes to give Compound **200** as a yellow powder.



Compound **200**

Example 62. Preparation of Compound **201**.

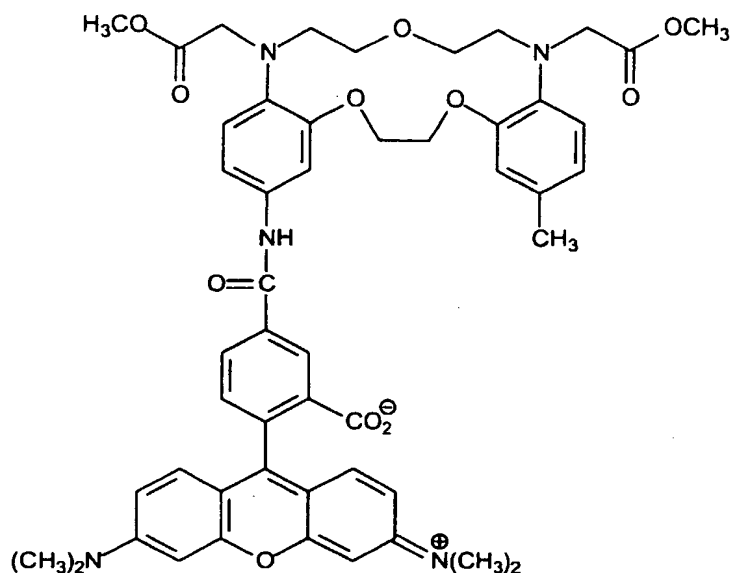
A 0.5 M solution of Compound **200** in methanol is treated with 10% palladium on charcaol, at 10 wt% of Compound **200**. The resulting mixture is shaken under 40 psi hydrogen until TLC analysis shows full conversion to aniline **201**. The reaction mixture is filtered through diatomaceous earth, followed by concentration in vacuo. The residue is trituated with ether-hexanes to give pure Compound **201** as an off-white powder.



Compound **201**

Example 63. Preparation of Compound **203**.

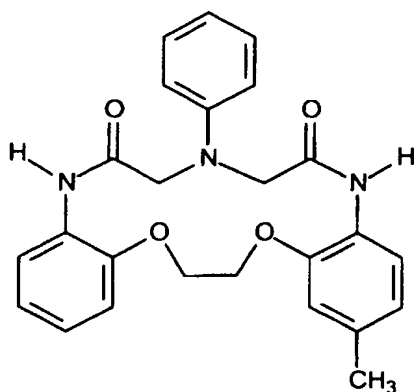
A mixed anhydride of 6-carboxytetramethylrhodamine is prepared according to the procedure given in U.S. Patent No. 5,453,517 to Kuhn et al. To a solution of the mixed anhydride and one equivalent of diisopropylethylamine in anhydrous THF under nitrogen is added slowly a 0.3 M solution of Compound **201**. The resulting mixture is stirred until TLC analysis indicates consumption of Compound **201**. The reaction mixture is concentrated in vacuo, and the residue partitioned between chl roform and water. The chl roform layer is washed with brine and dried over magnesium sulfate, then concentrated in vacuo. The residue is purified by flash chromatography on silica gel using increasing amounts of methanol in chloroform as solvent to give pure Compound **203** as a red powder.



Compound 203

Example 64. Preparation of a tris-aza crown ether (205).

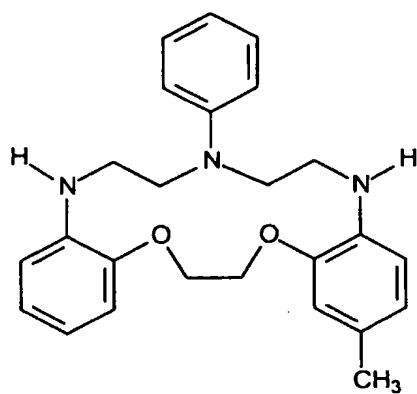
5 To a dilute solution (0.1 M) of diamine 105 and two equivalents of DIPEA in anhydrous THF is slowly added at room temperature a dilute solution (0.1 M) of N,N-bis(chlorocarbonylmethyl)-aniline. After stirring overnight, the volatiles are removed by evaporation, and the residue is partitioned between 5% hydrochloric acid and ethyl acetate. The organic layer is washed with
 10 brine, dried, and concentrated. The resulting residue is purified by column chromatography on silica gel using increasing amounts of methanol in chloroform to give pure Compound 205.



Compound 205

15 **Example 65. Preparation of Compound 206.**

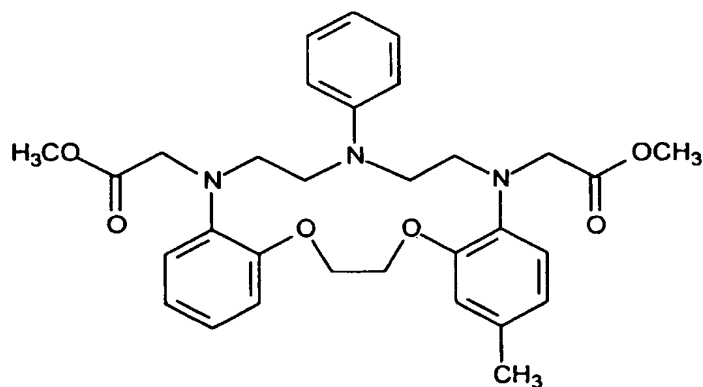
The bisamide 205 is reduced to bisaniline 206 as described for Compound 107.



Compound 206

Example 66. Preparation of Compound 207.

- 5 The bisaniline **206** is alkylated to give Compound **207**, as described for Compound **108**.

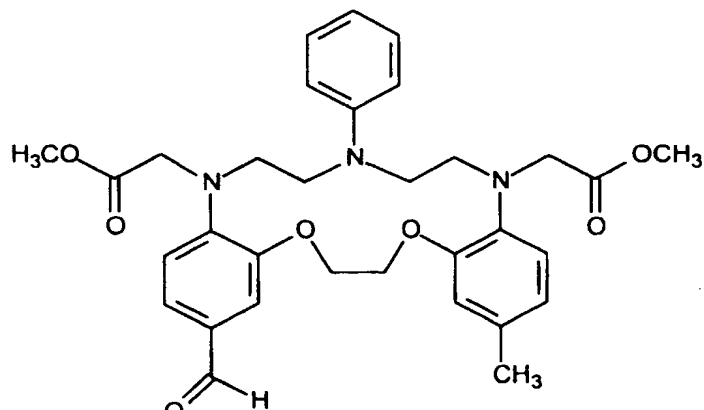


Compound 207

Example 67. Preparation of Compound 208.

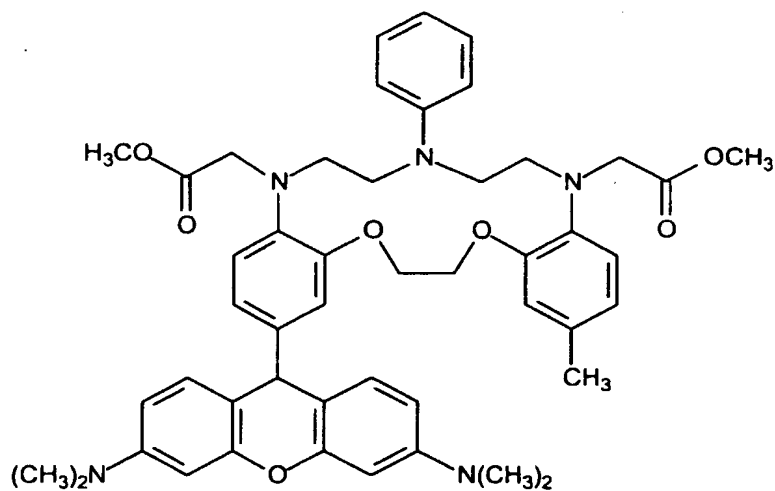
10

The bisester **207** is formylated to give the aldehyde Compound **208**, as described for Compound **109**.



Example 68. Preparation of Compound 209.

5 The aldehyde 208 is condensed with two equivalent of 3-(dimethylamino)phenol as described for Compound 137 to give Compound 209.

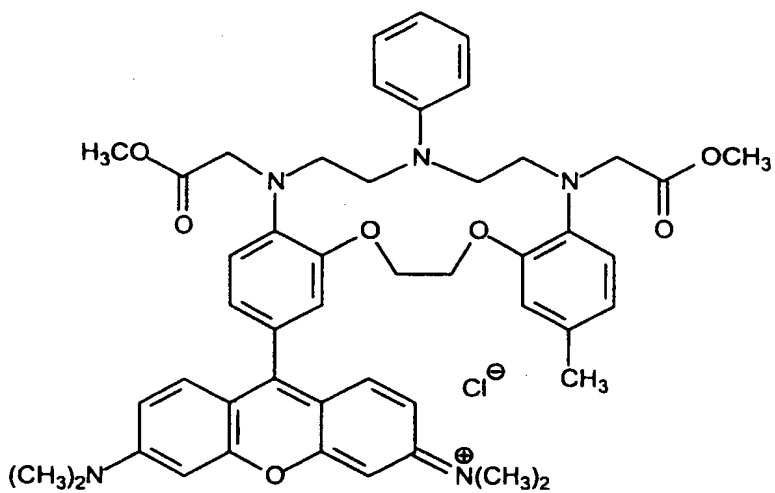


Compound 209

Example 69. Preparation of Compound 210.

10

Compound 209 is oxidized with chloranil to give the chloride salt 210 as a red powder, as described for Compound 138.



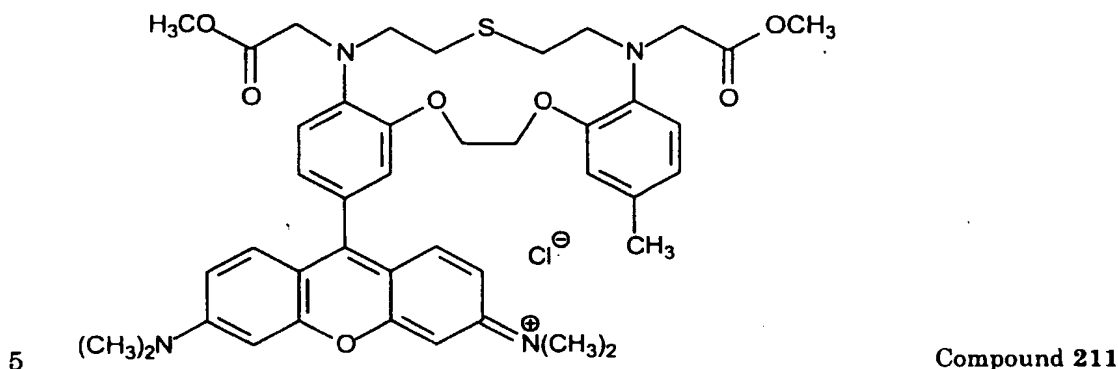
Compound 210

15

Example 70. Preparation of a thia-substituted crown ether (211).

A bis-aza crown ether that incorporates a sulfur atom in the crown is prepared using the procedures of Examples 64-69, except that in place of N,N-bis(chlorocarbonylmethyl)-aniline,

the bis-acid chloride of thiodiglycolic acid is used ($\text{Cl}(\text{C}=\text{O})\text{CH}_2\text{-S-CH}_2(\text{C}=\text{O})\text{Cl}$). After preparation of the crown ether itself, condensation with 3-(N,N-dimethylamino)phenol, and oxidation, the chloride salt of the thia-crown ether, Compound 211, is isolated as a red powder.



Example 71. Determination of sodium indicator fluorescence response.

10 The fluorescence response of a selected compound of the invention as a function of sodium ion concentration was determined by dissolving a sample of the pure compound in 3 mL of each of two solutions: solution 1 ("high Na^+ ") consists of 200 mM NaCl and 10 mM MOPS buffer at pH 7.05; solution 2 ("zero Na^+ ") consists of 10 mM MOPS buffer at pH 7.05 in deionized water. A series of curves are generated by cross dilution between the two solutions to arrive at intermediate concentrations of Na^+ . The emission of a selected indicator in solution 2 was

15 scanned and then was repeated to cover the entire range from zero to 200 mM Na^+ . For example, the fluorescence emission of the chosen indicator was scanned while the sample was excited at that indicator's absorption maximum wavelength, and then 1/100 of the sample was removed and replaced with 1/100 of solution 1 to arrive at a Na^+ concentration of 2 mM. This dilution was repeated to cover the entire range from zero to 1 M Na^+ and the resulting emission

20 intensities were plotted versus the ion concentrations. A least-squares fit was used to arrive at the concentration where the selected indicator was maximally sensitive to changes in Na^+ concentration. This corresponded to the dissociation constant of that indicator for Na^+ and was expressed as a concentration.

25 For visible wavelength probes such as Compound 132 (Example 17) and Compound 138 (Example 22) the indicator's fluorescence emission was typically scanned from 450-650 nm while the sample was excited at the absorption maximum wavelength. For UV-excitable ratiometric probes such as Compound 167 (Example 46), the excitation wavelengths of the dye in solution 2 were scanned from 260 to 450 nm while monitoring constant fluorescence emission at 510 nm (as

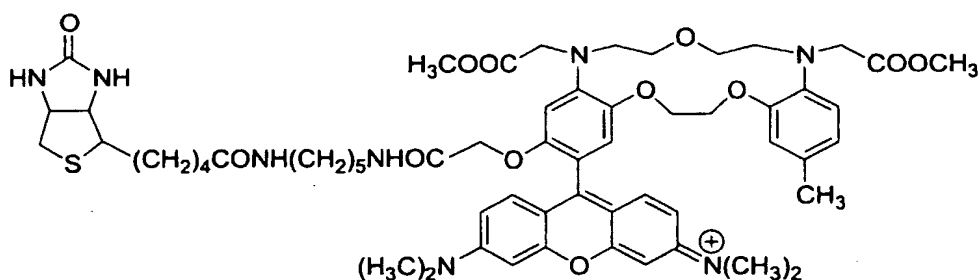
30 in Figure 1).

The binding affinity of a selected indicator for sodium ions, in the presence of potassium ions, is

Example 72. Calibration of sodium indicator fluorescence response in cells.

Jurkat cells were loaded with a 5 μ M solution of either Compound 135 (Example 20) or
5 commercially available SODIUM GREEN tetraacetate indicator (Molecular Probes, Inc.,
Eugene, OR) for 30 minutes at 37 °C. The use of PLURONIC^(R7m)/dispersing agent helped dissolve
the selected indicator. Intracellular sodium concentrations were then established by varying
the sodium concentration of the extracellular buffer in the presence of 2 μ M gramicidin (a
sodium-pore forming antibiotic). The extracellular buffer was set at 0 mM, 10 mM, 20 mM, 50
10 mM, 100 mM, and 145 mM, respectively. Intracellular fluorescence response of the selected
indicators was measured using a FACSCAN flow cytometer and associated software, with
fluorescence excitation at 488 nm. Compound 135 exhibits substantially brighter intracellular
fluorescence intensity than the SODIUM GREEN indicator at comparable Na⁺ concentrations,
as shown in Figure 3. Similarly, as intracellular Na⁺ concentration was increased, Compound
15 135 exhibits a consistent increase in fluorescence intensity.

Example 73. Preparation of a biotinylated indicator, Compound 212.



Compound 212

20 To a stirred solution of succinimidyl ester 155 prepared as in Example 36 from compound 153
(81 mg, 0.1 mmol) in DMF (2 mL) was added Et₃N (0.27 mL, 2 mmol) followed by biotin-
cadaverin trifluoroacetate (66 mg, 0.2 mmol). The mixture was stirred for 16 h, diluted with
CHCl₃ (100 mL), washed 1% AcOH (3 x 50 mL), H₂O (100 mL) dried over MgSO₄ and
25 evaporated. The residue was purified by preparative TLC on four C18 reverse-phase plates
using 50% aqueous 2-PrOH with 0.2% TFA as eluant to give Compound 212, 25 mg (22%) as
an orange solid.

Example 74. Avidin-labeling with a biotinylated fluorescent crown ether.

30 A 5 mg/mL solution of streptavidin in phosphate-buffered saline (PBS, pH 7.0) is treated with
a 1 mg/mL solution of biotinylated indicator (e.g., Compound 212, Example 73) in 2%

C

DMSO/PBS at a molar ratio such that two equivalents of biotinylated indicator are present for every streptavidin molecule. The resulting solution is incubated at 37°C for 4 hours, then centrifuged. The supernatant is applied to a Sephadex G-25 gel filtration column (2 mL bed volume/mg protein) and eluted with PBS. The streptavidin-indicator complex elutes first.

5 Fractions are analyzed by TLC to ensure that no free indicator is present in the complex. Pure product fractions are pooled and lyophilized. The complex is useful as a bridging method to apply the indicator to any biotinylated surface or substance.

10 All publications, patents and patent applications referred to within this document are incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

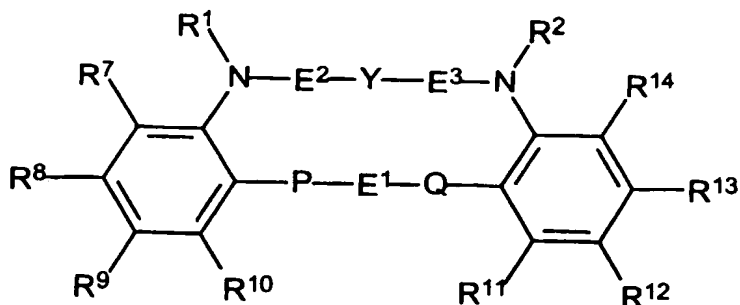
The reagents employed in the examples are commercially available or can be prepared using commercially available instrumentation, methods, or reagents known in the art. The foregoing examples illustrate various aspects of the invention and practice of the methods of the invention. The examples are not intended to provide an exhaustive description of the many different embodiments of the invention. Thus, although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, those of ordinary skill in the art will realize readily that many changes and modifications can be made thereto without departing from the scope of the appended claims.

15

20

CLAIMS

1. A compound of the formula



wherein

- 5 P and Q are independently O, S, or NR³, where each R³ is independently H or C₁-C₆ alkyl;
- Y is O, S, or NR⁴, where R⁴ is H; or is -L-R_x, -L-Sc, or -L-DYE; or is C₁-C₁₈ alkyl or an aryl or heteroaryl ring system, which alkyl or ring system is optionally substituted by halogen, azido, nitro, nitroso, amino, C₁-C₆ alkylamino, C₂-C₁₂ dialkylamino, cyano, -L-R_x, -L-Sc, or -L-DYE; or by C₁-C₆ alkyl or C₁-C₆ alkoxy that is itself optionally substituted by halogen, amino, hydroxy,
- 10 -(SO₂)-R¹⁵, -(SO₂)-O-R¹⁵, -(C=O)-R¹⁵, -(C=O)-O-R¹⁶, or -(C=O)-NR¹⁷R¹⁸; wherein

R¹⁵ is H or C₁-C₆ alkyl; or -L-R_x, -L-Sc, or -L-DYE;

R¹⁶ is H, a C₁-C₆ alkyl, a benzyl, a biologically compatible esterifying group, a biologically compatible salt; or -L-R_x, -L-Sc, or -L-DYE;

- 15 R¹⁷ and R¹⁸ are independently H, C₁-C₆ alkyl, C₁-C₆ carboxyalkyl, an alpha-acyloxyalkyl, a t-butyldimethylsilyl, or a biologically compatible salt; or -L-R_x, -L-Sc, or -L-DYE; or R¹⁷ and R¹⁸ taken in combination form a 5- or 6-membered aliphatic ring that optionally incorporates an oxygen atom;

each L is independently a covalent linkage;

each R_x is independently a reactive functional group;

- 20 each Sc is independently a conjugated substance;

DYE is a chemical moiety with an absorption maximum beyond 320 nm;

E¹, E², and E³ are independently -(CR⁵)_n-, or -(C(O)CH₂)_n-, where n = 2-4, and each R⁵ is independently H or CH₃, or two R⁵ moieties on adjacent carbons of one or more of E¹, E² or E³, when taken in combination, form a 5- or 6-membered aliphatic ring;

R^1 and R^2 are independently -L-R_x, -L-Sc, or -L-DYE; or C₁-C₁₈ alkyl or C₇-C₁₈ arylalkyl, each of which is optionally substituted by halogen, azido, nitro, nitroso, amino, hydroxy, cyano, or by an aryl or heteroaryl ring system; or by -(SO₂)-R¹⁵, -(SO₂)-O-R¹⁵, -(C=O)-R¹⁵, -(C=O)-O-R¹⁵, -(C=O)-NR¹⁷R¹⁸; or by C₁-C₆ alkylamino, C₂-C₁₂ dialkylamino; or by C₁-C₆ alkyl or C₁-C₆ alkoxy, each of which is itself optionally substituted by halogen, amino, hydroxy, -(SO₂)-R¹⁵, -(SO₂)-O-R¹⁵, -(C=O)-R¹⁵, -(C=O)-O-R¹⁵, -(C=O)-NR¹⁷R¹⁸;

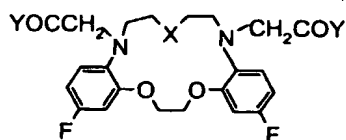
R^7 - R^{14} are independently H, halogen, azido, nitro, nitroso, amino, cyano, -L-R_x, -L-Sc, -L-DYE; or C₁-C₆ alkyl or C₁-C₆ alkoxy, each of which is itself optionally substituted by halogen, amino, hydroxy, -(SO₂)-R¹⁵, -(SO₂)-O-R¹⁵, -(C=O)-R¹⁵, -(C=O)-O-R¹⁵, or -(C=O)-NR¹⁷R¹⁸;

or any two adjacent substituents R^7 - R^{14} , taken in combination, form a fused six-membered benzo moiety, which is optionally substituted by halogen, azido, nitro, nitroso, amino, cyano, -L-R_x, -L-Sc, or -L-DYE; or C₁-C₆ alkyl or C₁-C₆ alkoxy, each of which is optionally substituted by halogen, amino, hydroxy, -(C=O)-R¹⁵, -(C=O)-O-R¹⁵, or -(C=O)-NR¹⁷R¹⁸;

or any two adjacent substituents R^7 - R^{14} , taken in combination with each other, and with the aromatic ring they are bound to, form a fused DYE;

provided that said compound is substituted by at least one -L-DYE, -L-R_x, or -L-Sc at R^1 , R^2 , R^4 , R^7 , R^8 , R^9 , R^{10} , R^{11} , R^{12} , R^{13} , or R^{14} ; or at least two of R^7 - R^{14} , taken in combination, form a fused DYE,

and provided that the compound is not of the formula



where Y is OH or OEt and X is -O-, or where Z is -NO₂, NH₂ or N(CH₂COY)₂.

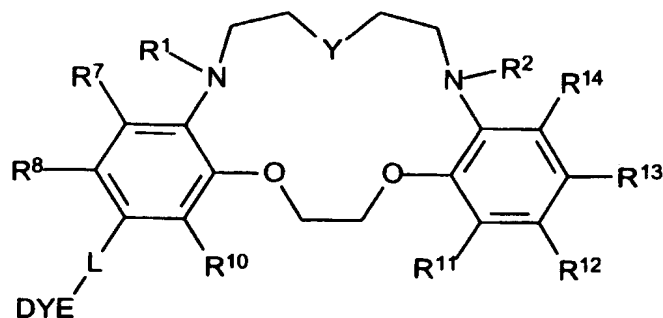
20

2. A compound, as claimed in Claim 1, wherein each R_x is independently an acrylamide, an activated ester of a carboxylic acid, an acyl azide, an acyl halide, an acyl nitrile, an aldehyde, a ketone, an alkyl halide, an alkyl sulfonate, an anhydride, an aniline, an aryl halide, an azide, an aziridine, a boronate, a carbodiimide, a diazoalkane, an epoxide, a haloacetamide, a halotriazine, a hydrazine, an imido ester, an isocyanate, an isothiocyanate, a maleimide, a phosphoramidite, a reactive platinum complex, a silyl halide, a sulfonate ester, a sulfonyl halide, a ps ral n, an aliphatic amine or a thiol.

25

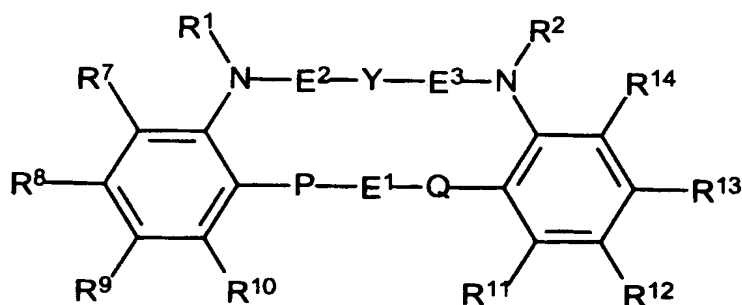
4. A compound, as claim d in any of Claims 1 to 3, wherein Y is NR⁴.
5. A compound, as claimed in Claim 1 wherein P and Q are O.
6. A compound, as claimed in Claim 5, wherein Y is O.
7. A compound, as claimed in Claim 6, wherein said compound is substituted by only one
5 -L-R_x, or -L-Sc, that is bound at R⁸, R⁹, R¹², or R¹³.
8. A compound, as claimed in any of Claims 1 to 7, wherein R¹ and R² are C₁-C₆ alkyl that are substituted one or more times by cyano, -(C=O)-O-R¹⁶, or -(C=O)-NR¹⁷R¹⁸.
9. A compound, as claimed in any of Claims 1 to 8, wherein R⁸ and R⁹, and optionally R¹² and R¹³, taken in combination, form a fused DYE that is a substituted or unsubstituted
10 benzofuran.
10. A compound, as claimed in any of Claims 1 to 9, wherein said compound is substituted by exactly two DYE or fused DYE moieties.
11. A compound, as claimed in any of Claims 1 to 8, wherein said compound is substituted by exactly one -L-DYE moiety at R⁹, and is optionally additionally substituted by exactly one
15 -L-R_x or exactly one -L-Sc.
12. A compound, as claimed in any of Claims 1 to 11, wherein each L is independently a single covalent bond, or a covalent linkage that is linear or branched, cyclic or heterocyclic, saturated or unsaturated, having 1-20 nonhydrogen atoms selected from the group consisting of C, N, P, O and S; and comprises any combination of ether, thioether, amine, ester,
20 carboxamide, sulfonamide, hydrazide bonds and aromatic or heteroaromatic bonds.
13. A compound, as claimed in Claim 12, wherein L is a single covalent bond or has the formula -(CH₂)_d(CONH(CH₂)_e)_z- or -O(CH₂)_d(CONH(CH₂)_e)_z-, where d is an integer from 0-5, e is an integer from 1-5 and z is 0 or 1.
14. A compound, as claimed in any of Claims 1 to 13, that is substituted by at least one Sc,
25 wherein each Sc is an amino acid, a peptide, a protein, a polysaccharide, a nucleoside, a nucleotide, an oligonucleotide, a nucleic acid, a hapten, a psoralen, a drug, a hormone, a lipid, a lipid assembly, a synthetic polymer, a polymeric microparticle, a biological cell or a virus.
15. A compound, as claimed in any of Claims 1 to 13, wherein said compound is substituted by exactly one Sc that is a protein, a peptide, a polysaccharide, a biotin, or a silica.
16. A compound, as claimed in any of Claims 1 to 15, that is substituted by at least one -L-R_x, wherein R_x is a succinimidyl ester of a carboxylic acid, a haloacetamide, a hydrazine, an isothiocyanate, a maleimide, an aliphatic amine, a silyl halide, or a psoralen.
30

24 10 02



wherein Y is O or NR⁴.

- 5 18. A compound, as claimed in Claim 17, wherein DYE is an indole, a coumarin, a stilbene, a xanthene, an oxazine, or a polyazaindacene.
19. A compound, as claimed in Claim 18, wherein DYE is a fluorescein, a rhodamine, a rhodol, a polyazaindacene, an oxazine, a 3*H*-xanthen-6-ol-3-one, a 6-amino-3*H*-xanthen-3-one, or a 6-amino-3*H*-xanthen-3-imine, and L is a single covalent bond.
- 10 20. A compound, as claimed in any of Claims 17 to 19, wherein R¹ and R² are C₁-C₆ alkyl that are substituted one or more times by -(C=O)-O-R¹⁶ or -(C=O)-NR¹⁷R¹⁸.
21. A compound, as claimed in Claim 20, wherein R¹ and R² are C₁-C₆ alkyl that are substituted one or more times by -(C=O)-O-R¹⁶, where each R¹⁶ is H, C₁-C₆ alkyl, an alpha-acyloxymethyl, a t-butyldimethyldimethylsilyl, or a biologically compatible salt.
- 15 22. A composition of matter comprising a compound of the formula:



wherein

P and Q are independently O, S, or NR³, where each R³ is independently H or C₁-C₆ alkyl;

Y is O, S, or NR⁴, where R⁴ is H; or is -L-R_X, -L-S_C, or -L-DYE; or is C₁-C₁₈ alkyl or an aryl or heteroararyl ring system, which alkyl or ring system may contain one or more substituents selected from:

azido, nitro, nitroso, amino, C₁-C₆ alkylamino, C₂-C₁₂ dialkylamino, cyano, -L-R_x, -L-Sc, or -L-DYE; or by C₁-C₆ alkyl or C₁-C₆ alkoxy that is itself optionally substituted by halogen, amino, hydroxy, -(SO₂)-R¹⁵, -(SO₂)-O-R¹⁵, -(C=O)-R¹⁵, -(C=O)-O-R¹⁶, or -(C=O)-NR¹⁷R¹⁸; wherein

5 R¹⁵ is H or C₁-C₆ alkyl; or -L-R_x, -L-Sc, or -L-DYE;

R¹⁶ is H, a C₁-C₆ alkyl, a benzyl, a biologically compatible esterifying group, a biologically compatible salt; or -L-R_x, -L-Sc, or -L-DYE;

10 R¹⁷ and R¹⁸ are independently H, C₁-C₆ alkyl, C₁-C₆ carboxyalkyl, an alpha-acyloxyalkyl, a t-butyldimethylsilyl, or a biologically compatible salt; or -L-R_x, -L-Sc, or -L-DYE; or R¹⁷ and R¹⁸ taken in combination form a 5- or 6-membered aliphatic ring that optionally incorporates an oxygen atom;

each L is independently a covalent linkage;

each R_x is independently a reactive functional group;

each Sc is independently a conjugated substance;

15 DYE is a chemical moiety with an absorption maximum beyond 320 nm;

E¹, E², and E³ are independently -(CR⁵)_n, where n = 2-4, and each R⁵ is independently H or CH₃, or two R⁵ moieties on adjacent carbons of one or more of E¹, E² or E³, when taken in combination, form a 5- or 6-membered aliphatic ring;

20 R¹ and R² are independently -L-R_x, -L-Sc, or -L-DYE; or C₁-C₁₈ alkyl or C₇-C₁₈ arylalkyl, each of which is optionally substituted by halogen, azido, nitro, nitroso, amino, hydroxy, cyano, or by an aryl or heteroaryl ring system; or by -(SO₂)-R¹⁵, -(SO₂)-O-R¹⁵, -(C=O)-R¹⁵, -(C=O)-O-R¹⁶, -(C=O)-NR¹⁷R¹⁸; or by C₁-C₆ alkylamino, C₂-C₁₂ dialkylamino; or by C₁-C₆ alkyl or C₁-C₆ alkoxy, each of which is itself optionally substituted by halogen, amino, hydroxy, -(SO₂)-R¹⁵, -(SO₂)-O-R¹⁵, -(C=O)-R¹⁵, -(C=O)-O-R¹⁶, -(C=O)-NR¹⁷R¹⁸;

25 R⁷-R¹⁴ are independently H, halogen, azido, nitro, nitroso, amino, cyano, -L-R_x, -L-Sc, -L-DYE; or C₁-C₆ alkyl or C₁-C₆ alkoxy, each of which is itself optionally substituted by halogen, amino, hydroxy, -(SO₂)-R¹⁵, -(SO₂)-O-R¹⁵, -(C=O)-R¹⁵, -(C=O)-O-R¹⁶, or -(C=O)-NR¹⁷R¹⁸;

30 or any two adjacent substituents R⁷-R¹⁴, taken in combination, form a fused six-membered benzo moiety, which is optionally substituted by halogen, azido, nitro, nitroso, amino, cyano, -L-R_x, -L-Sc, or -L-DYE; or C₁-C₆ alkyl or C₁-C₆ alkoxy, each of which is optionally substituted by halogen, amino, hydroxy, -(C=O)-R¹⁵, -(C=O)-O-R¹⁶, or -(C=O)-NR¹⁷R¹⁸;

or any two adjacent substituents R⁷-R¹⁴, taken in combination with each other, and with the aromatic ring they are bound to, form a fused DYE;

provided that said compound is substituted by at least one -L-DYE moiety at one or more of R¹, R², R⁴, and R⁷-R¹⁴; or at least two of R⁷-R¹⁴, taken in combination, form a fused DYE.

- 5 23. A composition, as claimed in Claim 22, wherein each R⁵ of the compound is H and each n is 2.
24. A composition, as claimed in Claim 22 or Claim 23, wherein each R_x of the compound is independently a reactive functional group that is an acrylamide, an activated ester of a carboxylic acid, an acyl azide, an acyl nitrile, an aldehyde, an alkyl halide, an anhydride, an aniline, an aryl halide, an azide, an aziridine, a boronate, a diazoalkane, a haloacetamide, a halotriazine, a hydrazine, an imido ester, an isocyanate, an isothiocyanate, a maleimide, a phosphoramidite, a reactive platinum complex, a silyl halide, a sulfonyl halide, or a thiol.
- 10 25. A composition, as claimed in any of Claims 22 to 24, wherein each P and Q on the compound are O.
- 15 26. A composition, as claimed in any of claims 22 to 24 or especially Claim 25, wherein each Y on the compound is O.
27. A composition, as claimed in any of claims 22 to 25 or especially Claim 26, wherein said compound is substituted by only one -L-R_x, or -L-Sc, that is bound at R⁸, R⁹, R¹², or R¹³.
- 20 28. A composition, as claimed in any of claims 22 to 24 or especially Claim 26 or Claim 27, wherein R¹ and R² are C₁-C₆ alkyl that are substituted one or more times by cyano, -(C=O)-O-R¹⁶, or -(C=O)-NR¹⁷R¹⁸.
29. A composition, as claimed in any of Claims 22 to 28, wherein R⁸ and R⁹, and optionally R¹² and R¹³, taken in combination, form a fused DYE that is a substituted or unsubstituted benzofuran.
- 25 30. A composition, as claimed in any of Claims 22 to 29, wherein said compound is substituted by exactly two DYE or fused DYE moieties.
31. A composition, as claimed in any of Claims 22 to 29, wherein said compound is substituted by exactly one -L-DYE moiety at R⁹, and is optionally additionally substituted by exactly one -L-R_x or exactly one -L-Sc.
- 30 32. A composition, as claimed in any of Claims 22 to 31, wherein each L of the compound is independently a single covalent bond, or a covalent linkage that is linear or branched, cyclic or heterocyclic, saturated or unsaturated, having 1-20 nonhydrogen atoms selected from the

group consisting of C, N, P, O and S; and comprise any combination of ether, thioether, amine, ester, carboxamide, sulfonamide, hydrazide bonds and aromatic or heteroaromatic bonds.

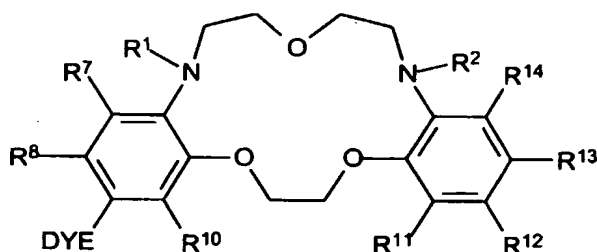
33. A composition, as claimed in Claim 32, wherein each L of the compound is a single covalent bond or has the formula $-(CH_2)_d(CONH(CH_2)_e)_z-$ or $-O(CH_2)_d(CONH(CH_2)_e)_z-$, where d is an integer from 0-5, e is an integer from 1-5 and z is 0 or 1.

34. A composition, as claimed in any of Claims 22 to 33, wherein each Sc of the compound is an amino acid, a peptide, a protein, a polysaccharide, a nucleoside, a nucleotide, an oligonucleotide, a nucleic acid, a hapten, a psoralen, a drug, a hormone, a lipid, a lipid assembly, a synthetic polymer, a polymeric microparticle, a biological cell or a virus.

35. A composition, as claimed in any of Claims 22 to 33, wherein said compound is substituted by exactly one Sc, which Sc is a protein, a polysaccharide, a biotin, or a silica.

36. A composition, as claimed in any of Claims 32 to 35, wherein said compound is substituted by exactly one Rx, which Rx is a succinimidyl ester of a carboxylic acid, a haloacetamide, a hydrazine, an isothiocyanate, a maleimide, an aliphatic amine, a silyl halide, or a psoralen.

37. A composition, as claimed in Claim 22 or as claimed in Claim 22 when combined with one or more of Claims 27 (when Y is O), 28 and 30 to 36, where the compound has the formula:



where R¹, R², R⁷, R⁸, and R¹⁰ are not -L-DYE, and no more than one, and optionally none, of R¹¹-R¹⁴ is -L-DYE.

38. A composition, as claimed in Claim 37, wherein each DYE on the compound is a fluorescein, a rhodamine, a rhodol, a polyazaindacene, an oxazine, a 3H-xanthen-6-ol-3-one, a 6-amino-3H-xanthen-3-one, or a 6-amino-3H-xanthen-3-imine.

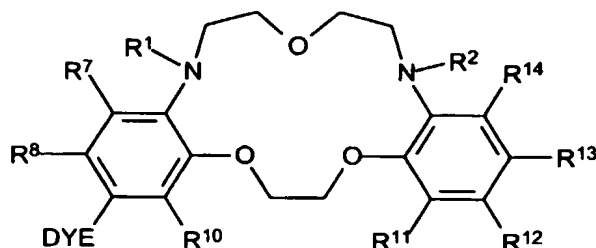
39. A composition, as claimed in Claim 37 or Claim 38, wherein R¹ and R² are C₁-C₆ alkyl that are substituted one or more times by $-(C=O)-O-R^{16}$ or $-(C=O)-NR^{17}R^{18}$.

40. A composition, as claimed in Claim 39, wherein R¹ and R² are C₁-C₆ alkyl that are substituted one or more times by $-(C=O)-O-R^{16}$, where each R¹⁶ is H, an alpha-acyloxymethyl, a t-butyldimethyldimethylsilyl, or a biologically compatible salt.

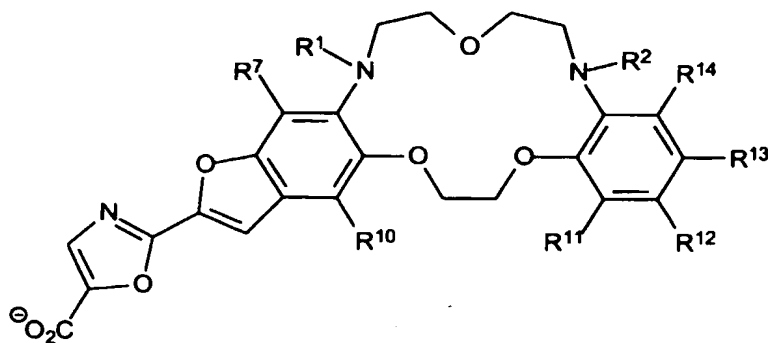
41. A composition, as claimed in any of Claims 22 to 36 or especially Claims 37 to 40, further comprising a metal ion that is Ca^{2+} , Na^+ , K^+ , or Zn^{2+} associated with said compound.

42. A composition, as claimed in any of Claims 22 to 41, further comprising a natural or synthetic polymer or glass.

5 43. A compound having the formula:



or the formula:



wherein

10 R^1 and R^2 are $\text{C}_1\text{-C}_6$ alkyl that are substituted one or more times by cyano, an aryl or heteroaryl ring system, or by $-(\text{C}=\text{O})\text{-O-R}^{16}$ or $-(\text{C}=\text{O})\text{-NR}^{17}\text{R}^{18}$, where

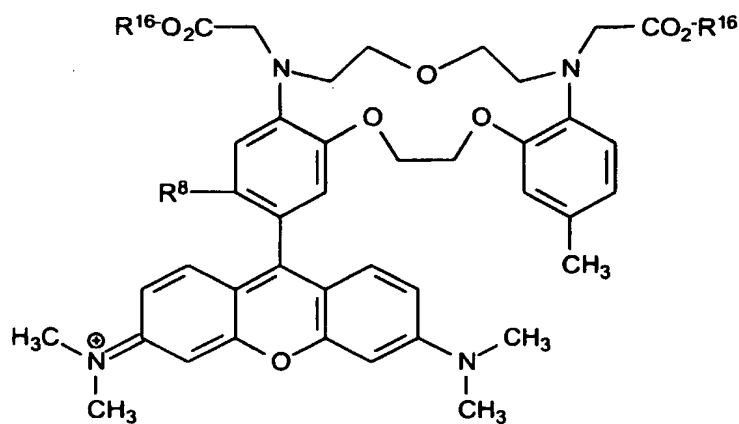
R^{16} is H, a $\text{C}_1\text{-C}_6$ alkyl, a benzyl, a biologically compatible esterifying group, or a biologically compatible salt;

15 R^{17} and R^{18} are independently H, $\text{C}_1\text{-C}_6$ alkyl, $\text{C}_1\text{-C}_6$ carboxyalkyl, an alpha-acyloxymethyl, or a biologically compatible salt;

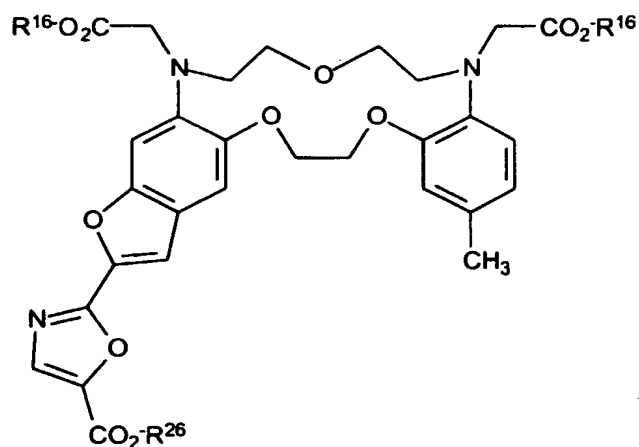
$\text{R}^7\text{-R}^{10}$, and $\text{R}^{11}\text{-R}^{14}$, where present, are independently H, chloro, bromo, fluoro, nitro, amino, or cyano; or $\text{C}_1\text{-C}_6$ alkyl or $\text{C}_1\text{-C}_6$ alkoxy that is itself optionally substituted by halogen, amino, hydroxy, $-(\text{SO}_2)\text{-R}^{15}$, $-(\text{SO}_2)\text{-O-R}^{15}$, $-(\text{C}=\text{O})\text{-R}^{15}$, $-(\text{C}=\text{O})\text{-O-R}^{16}$, or $-(\text{C}=\text{O})\text{-NR}^{17}\text{R}^{18}$; and

DYE, where present is a polyazaindacene, an oxazine, or a xanthene, which is optionally substituted by halogen, nitro, sulfo, cyano, an aryl or heteroaryl ring system, or benzo, r alkyl, perfluoroalkyl, alkoxy, alk nyl, alkynyl, cycloalkyl, arylalkyl, or acyl, the alkyl portions of which contain fewer than 20 carbons.

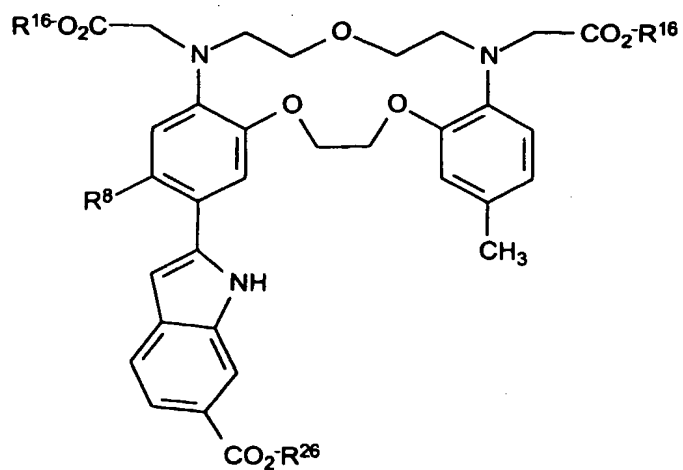
- 5 44. A compound having the formula:



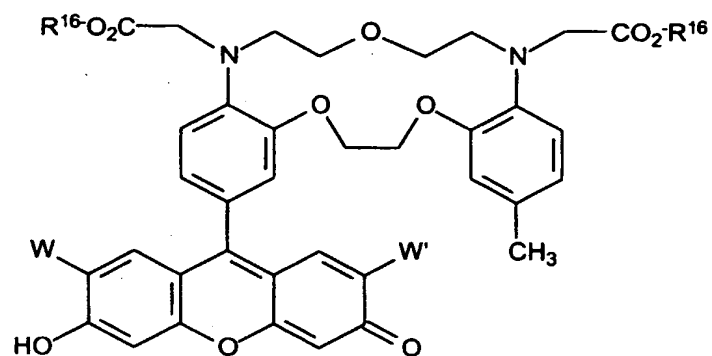
or the formula



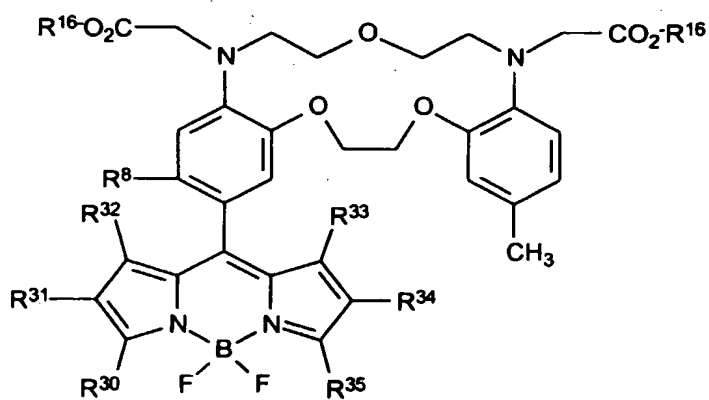
or the formula:



or the formula:



or the formula:



5

wherein

R⁸, where present, is independently H or a C₁-C₆ alkyl, which is optionally substituted by -(C=O)-O-R¹⁶ or -(C=O)-NR¹⁷R¹⁸;

R¹⁶ and R²⁶, where present, are independently H, a C₁-C₆ alkyl, a benzyl, an alpha-acyloxyalkyl, a t-butyldimethylsilyl, or a biologically compatible salt;

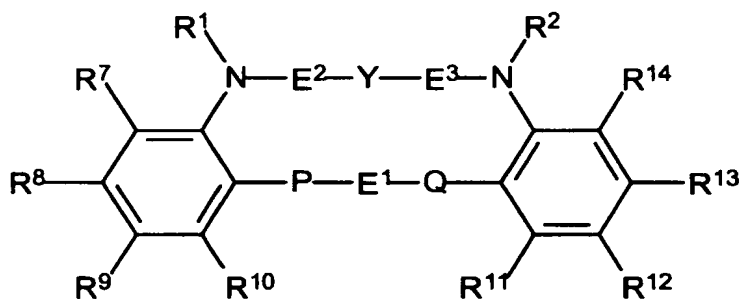
R¹⁷ and R¹⁸, where present, are independently H, a C₁-C₆ alkyl, C₁-C₆ carboxyalkyl, or a biologically compatible salt;

5 W and W', where present, are independently F or Cl;

R³⁰-R³⁵, where present, are independently H, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, or acyl, wherein the alkyl portions of each contain fewer than 20 carbons; or an aryl or heteroaryl ring system; or adjacent substituents R³¹ and R³², and adjacent substituents R³³ and R³⁴, when taken
10 in combination form a fused benzo ring that is optionally substituted by hydrogen, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, alkylthio, alkylamido, amino, monoalkylamino or dialkylamino wherein the alkyl portions of each contain fewer than 20 carbons.

45. A method of detecting a target cationic metal ion in a sample, comprising:

15 a) adding to said sample, in an amount sufficient to generate a detectable optical response when said target ion is present, a compound having the formula:



wherein the formula is as defined in any of Claims 22 to 42;

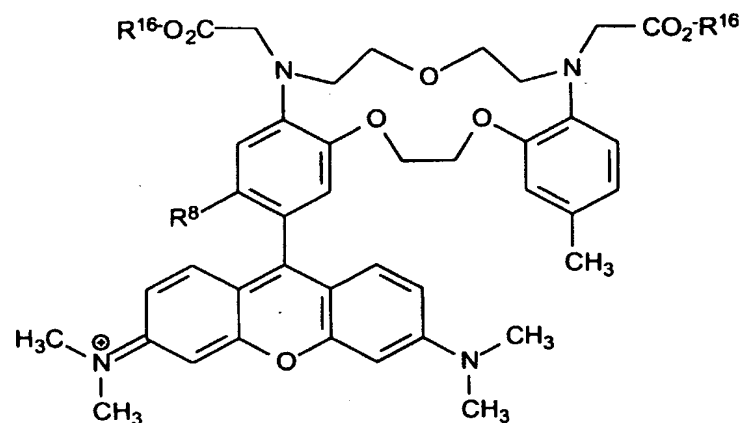
20 b) illuminating said sample to generate said detectable optical response that indicates that said target ion is present.

46. A method, as claimed in Claim 45, wherein said detectable optical response is a fluorescence response.

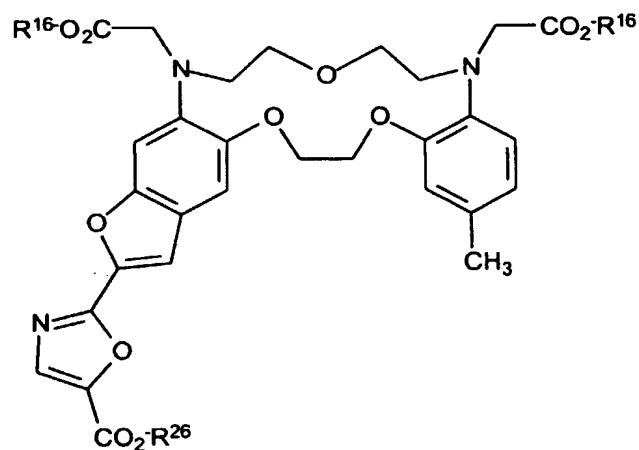
47. A method, as claimed in Claim 46, wherein the step of illuminating is performed in conjunction with a fluorometer, fluorescence microscope, laser scanner, flow cytometer, a
25 microfluidic device, or a fiber optic probe.

48. A method, as claimed in any of Claims 45 to 47, wherein said target metal ion is Na^+ , K^+ , Ca^{2+} , or Zn^{2+} .

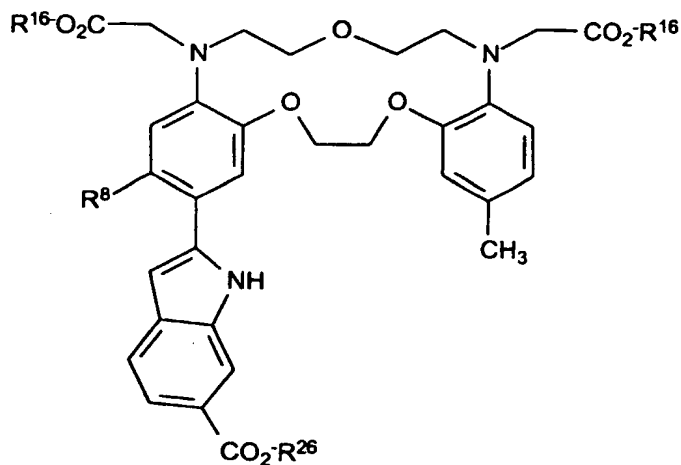
49. A method, as claimed in any of Claims 45 to 48, wherein said compound has the formula:



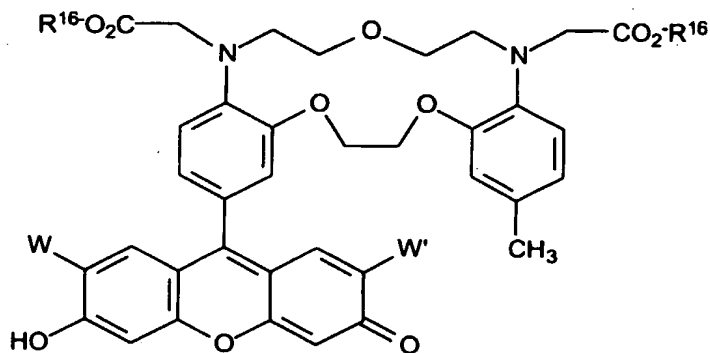
or the formula:



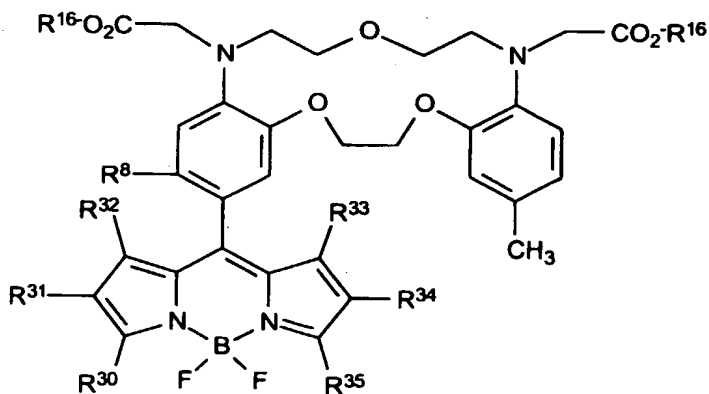
or the formula:



or the formula:



or the formula:



5

wherein

R^8 , where present, is independently H or a C_1 - C_6 alkoxy, which is optionally substituted by $-(C=O)-O-R^{16}$ or $-(C=O)-NR^{17}R^{18}$;

R^{16} and R^{26} , where present, are independently H, a C_1 - C_6 alkyl, a benzyl, an alpha-acyloxyalkyl, a t-butyldimethylsilyl, or a biologically compatible salt;

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R¹⁷ and R¹⁸, where present, are independently H, a C₁-C₆ alkyl, C₁-C₆ carboxyalkyl, or a biologically compatible salt;

W and W', where present, are independently F or Cl;

5 R³⁰-R³⁵, where present, are independently H, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, or acyl, wherein the alkyl portions of each contain fewer than 20 carbons; or an aryl or heteroaryl ring system.

50. A method, as claimed in Claim 49, wherein said target metal ion is Na⁺ or K⁺.

51. A method, as claimed in any of Claims 45 to 49, wherein said sample further comprises living cells or biological fluids.

10 52. A kit for the detection or quantification of a target metal ion, comprising a compound of any of Claims 1 to 21 or 43 or a composition of matter of any of Claims 22 to 42;

and comprising one or more components selected from the group consisting of:

a) a calibration standard of a target ion;

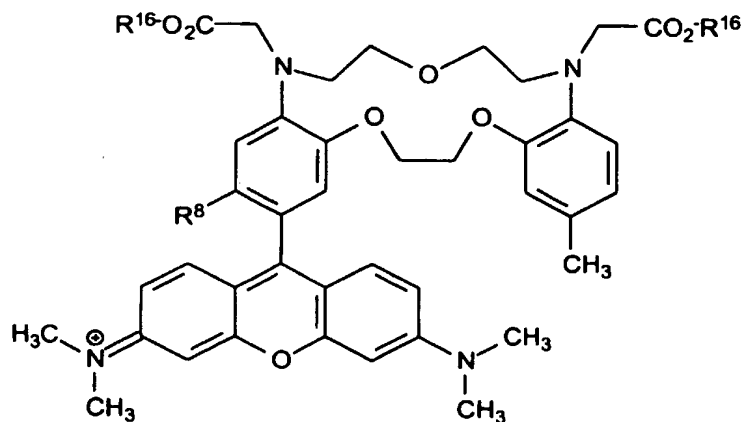
b) an ionophore;

15 c) a fluorescence standard;

d) an aqueous buffer solution; and

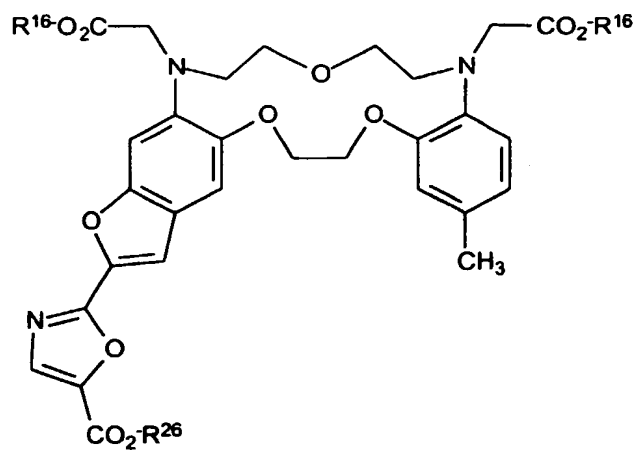
e) an organic solvent.

53. A kit for the detection or quantification of a target metal ion, comprising a compound having the formula:

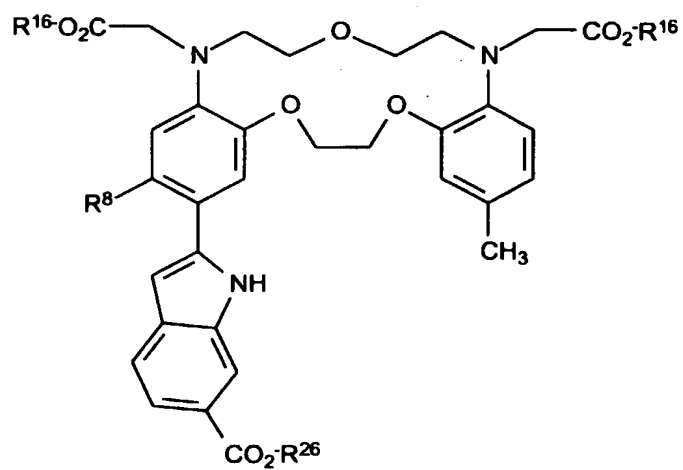


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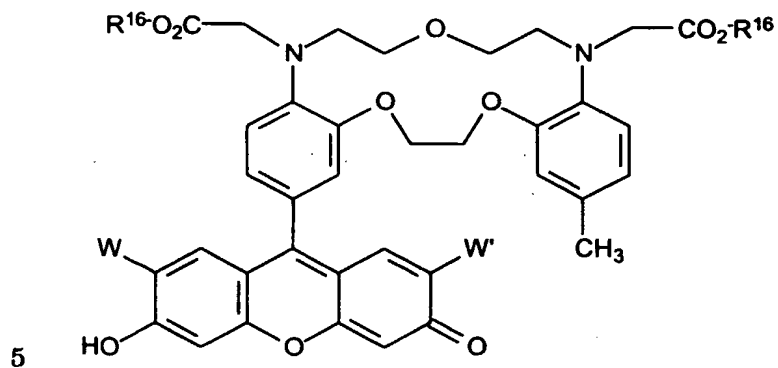
or the formula:



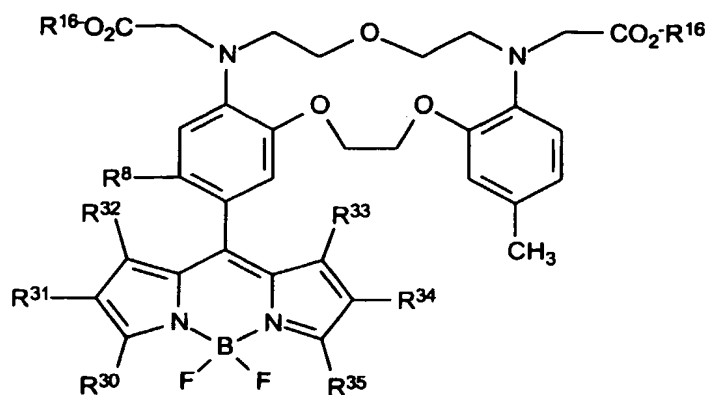
or the formula:



or the formula:



or the formula:



wherein

R⁸, where present, is independently H or a C₁-C₆ alkoxy, which is optionally substituted by -(C=O)-O-R¹⁶ or -(C=O)-NR¹⁷R¹⁸;

5 R¹⁶ and R²⁶, where present, are independently H, a C₁-C₆ alkyl, a benzyl, an alpha-acyloxyalkyl, a t-butyldimethylsilyl, or a biologically compatible salt;

R¹⁷ and R¹⁸, where present, are independently H, a C₁-C₆ alkyl, C₁-C₆ carboxyalkyl, or a biologically compatible salt;

W and W', where present, are independently F or Cl;

10 R³⁰-R³⁵, where present, are independently H, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, or acyl, wherein the alkyl portions of each contain fewer than 20 carbons; or an aryl or heteroaryl ring system;

and comprising one or more components selected from the group consisting of:

a) a calibration standard of a target ion;

15 b) an ionophore;

c) a fluorescence standard;

d) an aqueous buffer solution; and

e) an organic solvent.

54. A crown ether derivative of Claim 1 substantially as hereinbefore described or
20 exemplified.

55. The use of a compound of any of Claims 1 to 21, 43 or 44, a composition of matter of any of claims 22 to 42 or of a derivative of Claim 54 as a metal ion indicator.

56. A kit comprising a crown ether derivative of Claim 1 and substantially as hereinbefore described or exemplified.

57. A method of detecting or measuring a target cationic metal ion in a sample using a crown ether derivative of Claim 1 and substantially as hereinbefore described or exemplified.

5 58. A method of preparing a compound of any of Claims 1 to 21, 43 or 44, a composition of matter of any of claims 22 to 42 or of a derivative of Claim 54 and substantially as hereinbefore described or exemplified.

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